The Development and Application of a HPTLC-Derived Database for the Identification of Phenolics in Honey

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Abstract: This study reports on the development and validation of a HPTLC-derived database to identify phenolic compounds in honey. Two database sets are developed to contain the profiles of 107 standard compounds. Rich data in the form of Rf values, colour hues (H\(^{\circ}\)) at 254 nm and 366 nm, at 366 nm after derivatising with natural product PEG reagent, and at 366 nm and white light after derivatising with vanillin–sulfuric acid reagent, \(\lambda_{\text{max}}\) and \(\lambda_{\text{min}}\) values in their fluorescence and \(\lambda_{\text{max}}\) values in their UV-Vis spectra as well as \(\lambda_{\text{max}}\) values in their fluorescence and UV-Vis spectra after derivatisation are used as filtering parameters to identify potential matches in a honey sample. A spectral overlay system is also developed to confirm these matches. The adopted filtering approach is used to validate the database application using positive and negative controls and also by comparing matches with those identified via HPLC-DAD. Manuka honey is used as the test honey and leptosperine, mandelic acid, kojic acid, lepteridine, gallic acid, epigallocatechin gallate, 2,3,4-trihydroxybenzoic acid, o-anisic acid and methyl syringate are identified in the honey using the HPTLC-derived database.

Keywords: HPTLC; HPLC-DAD; Manuka honey; Leptospermum scoparium; database; biomarkers; phenolic compounds; phenolic determination

1. Introduction

Honey is an amber-coloured and viscous, natural substance produced by bees from the nectar of flowers (blossom honey) or the exudation of living parts of plants or insect excretions (honeydew honey) [1–3]. It is primarily composed of sugars (mostly glucose and fructose, which constitute approximately 60–85% of the honey’s total weight) and water (18–22%), as well as minor constituents (approximately 3%), such as amino acids, certain enzymes and other proteins, carotenoid-like substances, Maillard reaction products, minerals, vitamins, organic acids, phenolic acids and polyphenolic compounds, including flavonoids [1,3–6]. Honey is commonly considered a natural food supplement as some of the above-mentioned minor components can contribute not only to honey’s organoleptic characteristics [7], but also to its nutritional and health benefits [1,3–6]. Typically, honeys are offered as being either multifloral (produced by bees using nectar from many floral sources) or monofloral (derived from the nectar of predominantly one flower spices), and their botanical origin affects the quality and price [8].

Despite their relatively minor presence, phenolic compounds are one of the most studied honey constituents due to their well-known biological activities [7,9]. They are, furthermore, reported to influence the organoleptic characteristics of a honey, such as its colour, taste and aroma [3,7,8,10–12]. Phenolic compounds, such as flavonoids and phenolic acids, have also been identified as potential chemical markers for authenticating...
the geographical and botanical origin, and the quality of honey [1,4,13,14]. Moreover, they can also be used to monitor honey quality in order to choose the best processing practices [15]. A plethora of phenolic compounds have already been identified in a variety of honeys around the world [16]. The most frequently identified phenolics in honey belong to the class of hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives, flavonols, flavones and flavanones [16].

The regulation of honey quality is essential to ensure consumers receive a high-quality product at a price that is reflective of the botanical origin (e.g., monofloral/multifloral) and bioactivity levels of the honey [5,8,17]. Manuka honey, for example, is one of the most popular honeys worldwide due to its medicinal qualities, in particular, its exemplary antibacterial properties that can be correlated to its methylglyoxal levels (MGO) [18]. Because of this, the price of Manuka honey has been increasing, also leading to incidences of fraud and adulteration. In order to address this, the Ministry of Primary Industries (MPI), New Zealand, published a guideline for Manuka honey authentication by utilising 2′-methoxyacetophenone, (2′-MAPh), 2-methoxybenzoic acid or o-anisic acid, (2-MB), 3-phenyllactic acid (3-PLA) and 4-hydroxyphenyllactic acid (4-HPLA) as biomarker compounds [19]. An extensive analysis of phenolic compounds present in Manuka honey has been conducted by employing various chromatographic and spectroscopic techniques. HPLC using a photodiode array (DAD/PDA) or UV detectors is the most commonly used [20–30], followed by LC-MS [19,31–34], HPTLC [20] and fluorescence spectroscopy [35].

Using high-performance thin-layer chromatography (HPTLC), Stanek and Jasicka-Misiak in 2018 reported that Manuka honey samples contained rosmarinic acid, ellagic acid, p-coumaric acid and myricetin. The method was also employed in the phenolic-compound determination in other honeys, for which chlorogenic acid, caffeic acid, ferulic acid, 3,4-dihydroxybenzoic acid, abscisic acid and myricetin were identified in willow honey, while chlorogenic acid, caffeic acid, p-coumaric acid, abscisic acid, myricetin and chrysin were found in heather honey [20]. Furthermore, in 2020, Guzelmeric et al. identified caffeic acid as a biomarker for pine honey using HPTLC [5]. However, in these studies, only Rf values and colour were employed as the bases for compound matching, and no spectral scans were performed to confirm the identity of the compound. Furthermore, in these studies, only one derivatisation was used to derive the colour of the respective HPTLC bands.

HPTLC uses a high-grade stationary phase (commonly HPTLC-grade silica gel with a fluorescence indicator) alongside a suitable mobile-phase system, and the sample application is conducted semi-automatically. The instrumentation also includes a derivatiser that allows for the possible application of a suitable derivatisation reagent and generated images (typically at 254 and 366 nm prior to derivatisation, as well as at 366 nm and white light after derivatisation) are automatically captured by an imaging device. A TLC scanner, which can record the UV-Vis and fluorescence spectra of individual compound bands, complements the set up. An advantage of this analytical approach over others is the richness of the data generated, which includes not only Rf and peak intensity values for individual bands, but also their respective colours (recorded as RGB values) at various light conditions prior to and after derivatisation, as well as the possibility of capturing the UV-Vis and fluorescence spectra of individual compound bands, again prior to and after derivatisation with a suitable reagent. This study set out to develop and validate a HPTLC-derived database for the identification of phenolic compounds in honey. The database taps into the richness of HPTLC-generated data, beyond just Rf values and colour. Using this approach, it is anticipated that the identification of phenolic compounds that might act as marker compounds in a wide variety of honeys will add to the current suite of honey authentication methods.

2. Results
2.1. Database Development

To construct the four sub-databases (DB-1A, DB-1B, DB-2A, DB-2B), rich information was extracted from the HPTLC images of all 107 standards (Table 1).
| Name, Class and Code | Rf1 | Rf2 | 254 nm DEV H(•) and CE | 366 nm DEV H(•) and CE | 366 nm NP DER H(•) and CE | 366 nm VSA DER H(•) and CE | TVSA DER H(•) and CE | Fλ max and min | UV λ max | FNP λ | UVNP λ max | FVS λ | UV VS λ |
|---------------------|-----|-----|----------------|----------------|----------------|----------------|----------------|----------------|------------|--------|--------|-----------|--------|---------|
| 5-Methoxyflavone (5-MF), Flavone, (1) | 0.527 | 0.422 | 164 | 221 | 234 | 187 | 35 | 225, 254 | 268, 296, 331 | 245 | 297, 330 | 252 | 365 |
| 6-Hydroxyflavone-β-D-Glucoside (6-HF-β-D-Gluc.), Flavone, (2) | 0.108 | 0.035 | 155 | 237 | 242 | 203 | 31 | 224, 257 | 265 | 244 | 311 | 250 | 371 |
| Acacetin (Aca), Flavone, (3) | 0.680 | 0.564 | 135 | 180 | 78 | 204 | 54 | 224, 257 | 270, 329 | 244 | 360 | 249 | 365 |
| Apigenin (Api), Flavone, (4) | 0.647 | 0.507 | 134 | 199 | 109 | 206 | 46 | 224, 256 | 271, 336 | 243 | 361 | 249 | 365 |
| Baicalin (Bai), Flavone, (5) | 0.056 | 0.028 | 135 | 185 | 181 | 198 | 44 | 224, 246 | 284 | 243 | 304, 350 | 259 | 374 |
| Chrysirin (Chr), Flavone, (6) | 0.705 | 0.602 | 132 | 186 | 62 | 190 | 47 | 224, 255 | 270, 317 | 249 | 296, 341 | 249 | 383 |
| Genkwanin (Genk), Flavone, (7) | 0.657 | 0.533 | 139 | 46 | 126 | 229 | 47 | 224, 259 | 268, 338 | 243 | 367 | 249 | 399 |
| Luteolin (Lut), Flavone, (8) | 0.605 | 0.418 | 129 | 63 | 192 | 49 | 224, 253 | 268, 352 | 244 | 292, 460 | 251 | 376 |
| Vitexin (Vit), Flavone, (9) | 0.181 | 0.051 | 137 | 180 | 111 | 197 | 54 | 224, 262 | 273, 337 | 245 | 318, 365 | 249 | 373 |
| Fisetin (Fis), Flavonol, (10) | 0.568 | 0.368 | 138 | 176 | 31 | 184 | 52 | 224, 254 | 264, 325, 393 | 242 | 305, 338, 494 | 249 | 379 |
| Galangin (Gal), Flavonol, (11) | 0.737 | 0.642 | 131 | 172 | 172 | 194 | 42 | 224, 251 | 268, 311, 363 | 243 | 291, 441, 430 | 253 | 373 |
| Isorhamnetin (Isor), Flavonol, (12) | 0.665 | 0.536 | 136 | 180 | 167 | 191 | 43 | 224, 255 | 261, 364 | 250 | 293, 328, 434 | 252 | 388 |
| Kaempferide (Kaep), Flavonol, (13) | 0.713 | 0.616 | 129 | 144 | 161 | 188 | 45 | 223, 254 | 269, 330, 367 | 243 | 290, 365, 434 | 253 | 379 |
| Kaempferol (Kaem), Flavonol, (14) | 0.689 | 0.547 | 131 | 150 | 150 | 189 | 8 | 223, 254 | 269, 371 | 245 | 291, 369, 432 | 250 | 380 |
| Myricetin (Myr), Flavonol, (15) | 0.599 | 0.384 | 130 | 132 | 31 | 189 | 40 | 224, 253 | 261, 373 | 244 | 291, 488, 252 | 388 |
| Quercetin (Que), Flavonol, (16) | 0.636 | 0.207 | 126 | 127 | 37 | 192 | 41 | 224, 254 | 261, 370 | 239 | 291, 483 | 253 | 384 |
| Rutin (Rut), Flavonol, (17) | 0.041 | 0.022 | 133 | 180 | 42 | 189 | 43 | 224, 256 | 264, 351 | 238 | 291, 470 | 254 | 384 |
| Hesperetin (Hesper), Flavanone, (18) | 0.681 | 0.559 | 137 | 169 | 160 | 202 | 20 | 222, 238 | 290 | 249 | 339 | 250 | 375 |
| Hesperidin (Hespd), Flavanone, (19) | 0.070 | 0.019 | 140 | 186 | 24 | 214 | 33 | 223, 235 | 286, 329 | 247 | 328, 418 | 248 | 378 |
| Naringenin (Nar), Flavanone, (20) | 0.703 | 0.591 | 137 | 177 | 160 | 211 | 24 | 223, 246 | 292 | 249 | 333 | 250 | 365 |
| Name, Class and Code | Rf1 | Rf2 | 254 nm DEV H(°) and CE | 366 nm DEV H(°) and CE | 366 nm NP DER H(°) and CE | 366 nm VSA DER H(°) and CE | TVSA DER H(°) and CE | Fα max and min | UVα max | FNP | UVNPα max | FVSA | UVVSA |
|---------------------|-----|-----|------------------------|------------------------|------------------------|------------------------|------------------------|-----------------|----------|------|------------|------|-------|
| Naringin (Narg), Flavanone, (21) | 0.070 | 0.025 | 134 | 130 | 149 | 206 | 21 | 222, 236 | 286, 333 | 247, 328 | 422, 251 | 372 |
| Pinocembrin (Pinoc), Flavanone, (22) | 0.742 | 0.672 | 137 | 179 | 160 | 207 | 24 | 224, 241 | 293 | 246 | 338 | 251 | 370 |
| Sakuranetin (Sak), Flavanone, (23) | 0.721 | 0.623 | 137 | 160 | 102 | 200 | 16 | 223, 238 | 292 | 238 | 330 | 409 | 253 | 368 |
| Pinobanksin (Pinob), Flavanonol, (24) | 0.715 | 0.598 | 137 | 191 | 174 | 199 | 10 | 224, 239 | 295 | 245 | 340 | 253 | 365 |
| Taxifolin (Tax), Flavanonol, (25) | 0.594 | 0.359 | 135 | 183 | 51 | 206 | 24 | 224, 240 | 292 | 244 | 302 | 338 | 251 | 363 |
| Catechin (Cat), Flavan-3-ol, (26) | 0.533 | 0.256 | 138 | 142 | 85 | 211 | 18 | 224, 237 | 281 | 246 | 303 | 259 | 479 |
| Epicatechin (Epi), Flavan-3-ol, (27) | 0.517 | 0.228 | 137 | 138 | 135 | 209 | 11 | 224, 238 | 281 | 245 | 303 | 253 | 468 |
| Epigallocatechin (EGC), Flavan-3-ol, (28) | 0.460 | 0.130 | 137 | 177 | 111 | 201 | 2 | 225, 238 | 273 | 249 | 293 | 251 | 454 |
| Epigallocatechin Gallate (EGCG), Flavan-3-ol, (29) | 0.440 | 0.098 | 136 | 188 | 242 | 199 | 12 | 225, 240 | 282 | 246 | 332 | 249 | 495 |
| Biochanin A (Bio), Isoflavone, (30) | 0.711 | 0.603 | 133 | 97 | 156 | 195 | 28 | 224, 252 | 262 | 238 | 288 | 393 | 248 | 367 |
| Daidzein (Dai), Isoflavone, (31) | 0.616 | 0.422 | 137 | 161 | 226 | 226 | 29 | 222, 251 | 251 | 242 | 309 | 248 | 395 |
| Formononetin (For), Isoflavone, (32) | 0.663 | 0.517 | 147 | 195 | 194 | 179 | 38 | 223, 250 | 251 | 246 | 310 | 249 | 397 |
| Genistein (Gen), Isoflavone, (33) | 0.684 | 0.549 | 128 | 181 | 136 | 211 | 34 | 224, 253 | 260 | 249 | 289 | 395 | 248 | 419 |
| Genistin (Genist), Isoflavone, (34) | 0.211 | 0.072 | 133 | 177 | 148 | 200 | 26 | 222, 255 | 261 | 249 | 291 | 397 | 248 | 399 |
| t-Chalcone (t-Chal), Chalcone, (35) | 0.754 | 0.699 | 137 | 113 | 79 | 231 | 40 | 221, 257 | 310 | 232 | 308 | 249 | 361 |
| 2,3,4-Trimethoxybenzoic Acid (2,3,4-TMBA), HBAD, (36) | 0.629 | 0.504 | 138 | 182 | 171 | 226 | 21 | 223, 256 | 251 | 246 | 308 | 249 | 361 |
| 2,4,5-Trimethoxybenzoic Acid (2,4,5-TMBA), HBAD, (37) | 0.615 | 0.428 | 138 | 191 | 217 | 222 | 33 | 225, 251 | 251 | 239 | 308 | 248 | 410 |
| Benzoic Acid (BA), HBAD, (40) | 0.696 | 0.570 | 136 | 185 | 159 | 199 | 48 | 211, 241 | 276 | 316 | 246 | 277 | 251 | 371 |
| Cuminic Acid (CuA), HBAD, (41) | 0.710 | 0.525 | 136 | 180 | 185 | 205 | 2 | 221, 246 | 243 | 223 | 252 | 253 | 247 |
| Ellagic Acid (Ella), HBAD, (42) | 0.017 | 0.020 | 130 | 212 | 207 | 221 | 40 | 223, 253 | 277 | 389 | 248 | 291 | 318 | 383 |
| Eudesmic Acid (EudA), HBAD, (43) | 0.629 | 0.504 | 139 | 180 | 203 | 219 | 41 | 228, 254 | 264 | 232 | 291 | 248 | 252 |
Table 1. Cont.

| Name, Class and Code | Rf1 | Rf2 | 254 nm DEV H(◦) and CE | 366 nm DEV H(◦) and CE | 366 nm NP DER H(◦) and CE | 366 nm VSA DER H(◦) and CE | TVSA DER H(◦) and CE | Fa max and min (◦) | UVλ max | FNP λ | UVNP λ max | FVS λ | UV VS λ |
|---------------------|-----|-----|------------------------|------------------------|------------------------|------------------------|------------------------|-------------------|---------|-------|-------------|-------|--------|
| Gallic Acid (GA), HBAD, (44) | 0.544 | 0.321 | 135 | 152 | 256 | 217 | 43 | 225, 257 | 272 | 245 | 322 | 249 | 389 |
| Gentisic Acid (GenA), HBAD, (45) | 0.634 | 0.508 | 143 | 224 | 246 | 213 | 49 | 224, 246 | 326 | 241 | 352 | 247 | 355 |
| Leptosperine (Leps), HBAD, (46) | 0.017 | 0.012 | 137 | 188 | 207 | 223 | 45 | 229, 257 | 265 | 230 | 292 | 248 | 399 |
| Methyl Paraben (Mpar), HBAD, (47) | 0.683 | 0.573 | 128 | 179 | 220 | 228 | 111 | 224, 252 | 257 | 238 | 263 | 247 | 251 |
| Methyl Syringate (MS), HBAD, (48) | 0.634 | 0.524 | 138 | 175 | 203 | 219 | 43 | 237, 258 | 277 | 241 | 291 | 248 | 351 |
| Methyl-3,4,5-trimethoxybenzoate (M-3,4,5-TMBz), HBAD, (49) | 0.688 | 0.589 | 141 | 186 | 120 | 202 | 48 | 230, 256 | 265 | 239 | 294 | 245 | 432 |
| m-Hydroxybenzoic Acid (m-HBA), HBAD, (50) | 0.649 | 0.499 | 138 | 181 | 184 | 232 | 38 | 223, 245 | 298 | 253 | 298 | 251 | 422 |
| m-Toluic Acid (m-TA), HBAD, (51) | 0.675 | 0.617 | 137 | 170 | 169 | 206 | 25 | 221, 242 | 284 | 243 | 287 | 249 | 349 |
| o-Anisic Acid (o-AA), HBAD, (52) | 0.610 | 0.499 | 138 | 206 | 193 | 225 | 43 | 221, 243 | 299 | 243 | 295 | 248 | 452 |
| o-Toluic Acid (o-TA), HBAD, (53) | 0.662 | 0.633 | 138 | 175 | 215 | 222 | 33 | 221, 240 | 281 | 255 | 293 | 248 | 437 |
| p-Hydroxybenzoic Acid (p-HBA), HBAD, (54) | 0.656 | 0.513 | 130 | 184 | 180 | 212 | 28 | 225, 252 | 257 | 232 | 261 | 245 | 445 |
| Protocatechuic Acid (PrA), HBAD, (55) | 0.595 | 0.398 | 136 | 183 | 246 | 214 | 46 | 225, 255 | 261 | 295 | 242 | 326 | 249 | 474 |
| Resorcylic Acid (ReA), HBAD, (56) | 0.646 | 0.504 | 144 | 214 | 226 | 202 | 57 | 224, 249 | 251 | 313 | 241 | 331 | 254 | 348 |
| Salicylic Acid (SA), HBAD, (57) | 0.685 | 0.582 | 140 | 211 | 181 | 205 | 48 | 223, 242 | 301 | 254 | 326 | 247 | 369 |
| Syringic Acid (SyA), HBAD, (58) | 0.601 | 0.444 | 137 | 192 | 215 | 218 | 28 | 225, 258 | 277 | 240 | 290 | 247 | 351 |
| Vanillic Acid (VA), HBAD, (59) | 0.643 | 0.504 | 136 | 192 | 209 | 218 | 32 | 228, 255 | 263 | 294 | 240 | 295 | 251 | 492 |
| Vanillic Acid Methyl Ester (VAME), HBAD, (60) | 0.671 | 0.570 | 135 | 179 | 231 | 207 | 71 | 225, 254 | 263 | 294 | 243 | 297 | 248 | 423 |
| Caffeic Acid (CaA), HCAD, (61) | 0.597 | 0.421 | 139 | 211 | 197 | 215 | 38 | 224, 250 | 321 | 254 | 374 | 248 | 358 |
| Caffeic Acid Dimethyl Ether (CADE), HCAD, (62) | 0.625 | 0.501 | 142 | 239 | 223 | 212 | 101 | 223, 247 | 317 | 238 | 308 | 248 | 350 |
| o-Toluic Acid (o-TA), HBAD, (53) | 0.662 | 0.633 | 138 | 175 | 215 | 222 | 33 | 221, 240 | 281 | 255 | 293 | 248 | 437 |
| p-Hydroxybenzoic Acid (p-HBA), HBAD, (54) | 0.656 | 0.513 | 130 | 184 | 180 | 212 | 28 | 225, 252 | 257 | 232 | 261 | 245 | 445 |
| Protocatechuic Acid (PrA), HBAD, (55) | 0.595 | 0.398 | 136 | 183 | 246 | 214 | 46 | 225, 249 | 251 | 313 | 241 | 331 | 254 | 348 |
Table 1. Cont.

| Name, Class and Code | R1 | R2 | 254 nm DEV H(+) and CE | 366 nm DEV H(+) and CE | 366 nm NP DER H(+) and CE | 366 nm VSA DER H(+) and CE | TVSA DER H(+) and CE | Fλ max and min | UV λ max | FNP λ | UVNP λ max | FVS λ | UV VS λ |
|----------------------|----|----|------------------------|------------------------|--------------------------|--------------------------|------------------------|----------------|----------|--------|------------|--------|--------|
| Salicylic Acid (SA), HBAD, (57) | 0.685 | 0.582 | 140 | 211 | 181 | 205 | 48 | 223 | 242 | 301 | 254 | 326 | 247 | 369 |
| Syringic Acid (SyA), HBAD, (58) | 0.601 | 0.444 | 137 | 192 | 215 | 218 | 28 | 225 | 258 | 277 | 240 | 290 | 247 | 351 |
| Vanillic Acid (VA), HBAD, (59) | 0.643 | 0.504 | 136 | 192 | 209 | 218 | 32 | 228 | 255 | 263 | 294 | 240 | 295 | 251 | 492 |
| Vanillic Acid Methyl Ester (VAME), HBAD, (60) | 0.671 | 0.570 | 135 | 179 | 231 | 207 | 71 | 225 | 254 | 263 | 294 | 243 | 297 | 248 | 423 |
| Caffeic Acid (CaA), HCAD, (61) | 0.597 | 0.421 | 139 | 211 | 197 | 215 | 38 | 224 | 250 | 321 | 254 | 374 | 248 | 358 |
| Caffeic Acid Dimethyl Ether (CADE), HCAD, (62) | 0.625 | 0.501 | 142 | 239 | 223 | 212 | 101 | 223 | 247 | 317 | 238 | 308 | 248 | 350 |
| Caffeic Acid Phenetyl Ester (CAPE), HCAD, (63) | 0.684 | 0.554 | 140 | 235 | 209 | 240 | 27 | 225 | 250 | 325 | 254 | 392 | 249 | 411 |
| Chlorogenic Acid (ChlA), HCAD, (64) | 0.112 | 0.033 | 138 | 196 | 201 | 224 | 43 | 222 | 250 | 329 | 249 | 379 | 249 | 356 |
| Ferulic Acid (FA), HCAD, (65) | 0.636 | 0.508 | 139 | 209 | 206 | 215 | 41 | 224 | 246 | 319 | 239 | 317 | 248 | 353 |
| Isoferulic Acid (IFA), HCAD, (66) | 0.609 | 0.459 | 140 | 227 | 216 | 215 | 136 | 224 | 248 | 320 | 239 | 317 | 249 | 350 |
| m-Coumaric Acid (m-CA), HCAD, (67) | 0.644 | 0.493 | 143 | 215 | 220 | 208 | 49 | 224 | 252 | 280 | 228 | 288 | 252 | 344 |
| Methyl Ferulate (MF), HCAD, (68) | 0.682 | 0.576 | 138 | 236 | 228 | 214 | 83 | 224 | 250 | 324 | 233 | 327 | 251 | 354 |
| Neochlorogenic Acid (NChlA), HCAD, (69) | 0.057 | 0.014 | 141 | 228 | 217 | 205 | 48 | 224 | 256 | 329 | 250 | 382 | 248 | 365 |
| o-Coumaric Acid (o-CA), HCAD, (70) | 0.655 | 0.519 | 146 | 205 | 204 | 223 | 41 | 224 | 258 | 279 | 321 | 253 | 318 | 248 | 427 |
| p-Coumaric Acid (p-CA), HCAD, (71) | 0.644 | 0.510 | 136 | 199 | 205 | 207 | 41 | 223 | 258 | 307 | 255 | 298 | 248 | 348 |
| p-m-Cinnamic Acid (p-MCA), HCAD, (72) | 0.670 | 0.556 | 137 | 191 | 131 | 245 | 10 | 222 | 257 | 307 | 244 | 296 | 249 | 425 |
| Rosmarinic Acid (RosA), HCAD, (73) | 0.692 | 0.259 | 131 | 175 | 134 | 226 | 43 | 224 | 248 | 307 | 242 | 307 | 389 | 249 | 437 |
| Sinapic Acid (SinA), HCAD, (74) | 0.533 | 0.439 | 139 | 224 | 198 | 224 | 46 | 222 | 245 | 328 | 243 | 320 | 248 | 359 |
| t-Cinnamic Acid (t-CA), HCAD, (75) | 0.586 | 0.596 | 140 | 242 | 222 | 202 | 44 | 229 | 261 | 279 | 242 | 289 | 248 | 391 |
| Trans-p-Coumaric Acid Methyl Ester (t-p-CAME), HCAD, (76) | 0.697 | 0.584 | 136 | 193 | 202 | 205 | 49 | 222 | 237 | 309 | 245 | 314 | 250 | 437 |
| 3,4-Dihydroxyphenylacetic Acid (3,4-DHPAA), HPAAD, (77) | 0.592 | 0.365 | 137 | 180 | 235 | 270 | 11 | 224 | 238 | 283 | 242 | 305 | 250 | 422 |
| Homogentisic Acid (HGA), HPAAD, (78) | 0.569 | 0.348 | 139 | 184 | 132 | 237 | 49 | 224 | 235 | 294 | 253 | 298 | 250 | 396 |
| Homovanillic Acid (HVA), HPAAD, (79) | 0.609 | 0.441 | 137 | 177 | 213 | 230 | 57 | 222 | 240 | 282 | 244 | 288 | 248 | 452 |
| Name, Class and Code | R1 | R2 | Fλ max and min | UVλ max | FNP λ | UVNP λ max | FV λ | UV V λ |
|----------------------|----|----|----------------|--------|-------|------------|------|--------|
| Mandelic Acid (ManA), HPAAD, (80) | 0.530 | 0.347 | 139 | 165 | 208 | 235 | 223, 241 |
| Phenylacetic Acid (PAA), HPAAD, (81) | 0.678 | 0.589 | 136 | 175 | 343 | 242 | 23, 239 |
| p-Hydroxyphenylacetic Acid (p-HPAA), HPAAD, (82) | 0.643 | 0.471 | 138 | 179 | 180 | 265 | 11, 222 |
| L-β-Phenyllactic Acid (L-β -PLA), HPLAD, (83) | 0.701 | 0.640 | 139 | 186 | 226 | 220 | 26, 23, 23 |
| DL-p-Phenyllactic Acid (DL-p-HPLA), HPLAD, (84) | 0.636 | 0.466 | 139 | 120 | 171 | 244 | 25, 222 |
| 3-Phenylpropanoic Acid (3-PPA), HPPAD, (85) | 0.665 | 0.536 | 138 | 179 | 192 | 228 | 38, 222 |
| Phloretic Acid (PhlA), HPPAD, (86) | 0.650 | 0.483 | 141 | 188 | 190 | 229 | 33, 23, 23 |
| 2-Methoxy-4-vinylphenol (2-M4-VPh), AMPh, (87) | 0.707 | 0.631 | 122 | 189 | 191 | 308 | 339, 224 |
| Acetaminophen (Acet), p-AmPh, (92) | 0.486 | 0.295 | 121 | 171 | 183 | 226 | 37, 23, 23 |
| Pyrogallol (Pyrog), Phenol, (94) | 0.624 | 0.445 | 137 | 186 | 51 | 224 | 21, 222 |
| Protocatechualdehyde (PrCatd), HBzd, (98) | 0.619 | 0.453 | 135 | 232 | 233 | 240 | 29, 222 |
| Vanillin (Van), HBzd, (99) | 0.659 | 0.548 | 136 | 222 | 224 | 212 | 42, 221 |
| 2′-Hydroxyacetophenone (2′-HAPhn), HAPhn, (100) | 0.717 | 0.672 | 133 | 152 | 180 | 206 | 31, 228 |
| 2-Methoxyacetophenone (2′-MAPhn), HAPhn, (101) | 0.692 | 0.628 | 139 | 241 | 204 | 188 | 0, 222 |
| Dibenzyl Oxalate (DO), OE, (102) | 0.639 | 0.531 | 139 | 199 | 278 | 245 | 62, 222 |
### Table 1. Cont.

| Name, Class and Code | Rf1 | Rf2 | 254 nm DEV H<sup>+</sup> and CE | 366 nm DEV H<sup>+</sup> and CE | 366 nm NP DER H<sup>+</sup> and CE | 366 nm VSA DER H<sup>+</sup> and CE | 366 nm T VSA DER H<sup>+</sup> and CE | UV λ<sub>max</sub> | FNP λ<sub>max</sub> | UV NP λ<sub>max</sub> | Fl λ<sub>max</sub> and min | UV-Vis λ<sub>max</sub> | Fl λ<sub>max</sub> | UV-Vis λ<sub>max</sub> | Fl λ<sub>max</sub> | UV-Vis λ<sub>max</sub> |
|---------------------|-----|-----|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|----------------|----------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| Abisic Acid (AbsA), Non-phenolic, (103) | 0.612 | 0.435 | 127 | 171 | 205 | 340 | 356 | 221 | 251 | 268 | 223 | 265 | 250 | 385 |
| Benzophenone (Benzph), Non-phenolic, (104) | 0.737 | 0.652 | 120 | 171 | 196 | 240 | 40 | 224 | 253 | 260 | 235 | 261 | 288 | 239 | 251 |
| Kojic Acid (KojA), Non-Phenolic, (105) | 0.308 | 0.171 | 136 | 217 | 228 | 231 | 45 | 221 | 253 | 277 | 245 | 306 | 249 | 420 |
| Lepteridine (Leptd), Non-Phenolic, (106) | 0.322 | 0.217 | 148 | 195 | 209 | 242 | 21 | 227 | 249 | 329 | 247 | 332 | 248 | 365 |
| Lumichrome (Lum), Non-Phenolic, (107) | 0.482 | 0.326 | 153 | 209 | 221 | 216 | 52 | 225 | 257 | 261 | 297 | 249 | 361 | 249 | 391 |

Legend: Rf1—retention factor in MPA, Rf2—retention factor in MPB, 254 nm DEV H<sup>+</sup> and C—hue and colour equivalent at 254 nm prior to derivatisation, 366 nm DEV H<sup>+</sup> and C—hue and colour equivalent at 366 nm prior to derivatisation, 366 nm NP H<sup>+</sup> and C—hue and colour equivalent at 366 nm after derivatisation with NP-PEG derivatisation reagent, 366 nm VSA H<sup>+</sup> and C—hue and colour equivalent at 366 nm after derivatisation with VSA-derivatisation reagent, T VSA H<sup>+</sup> and C—hue and colour equivalent at transmittance in white light after derivatisation with VSA-derivatisation reagent, Fl DEV λ<sub>max</sub> and λ<sub>min</sub>—fluorescence λ<sub>max</sub> and λ<sub>min</sub> prior to derivatisation, UV DEV λ<sub>max</sub>—UV-Vis λ<sub>max</sub> prior to derivatisation, Fl NP λ<sub>max</sub>—fluorescence λ<sub>max</sub> after derivatisation with NP-PEG reagent, UV-Vis λ<sub>max</sub> after derivatisation with NP-PEG reagent, Fl VS λ<sub>max</sub>—fluorescence λ<sub>max</sub> after derivatisation with VSA reagent, UV-Vis λ<sub>max</sub> after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate. Subclass (see Tables S1–S14): HCAD—hydroxycinnamic acid and derivatives, HBAD—hydroxybenzoic acid and derivatives, HPPAD—hydroxyphenylpropionic acid and derivatives, AMPh—alkylmethoxyphenol, APh—alkylphenol, p-AmPh—p-aminophenol, HBzd—hydroxybenzaldehyde and derivatives, HAPh—hydroxyacetophenone and derivatives, OE—oxalate ester, NP—non-phenolics

#### 2.1.1. Retention Factor (Rf1 and Rf2 in MPA and MPB, respectively)

MPB was selected as the mobile phase because prior studies of honey using HPTLC analysis employed this mobile phase, allowing for cross-references to previous work. MPA, with slightly higher polarity, was chosen to ensure that more polar phenolics were also adequately separated and detected [36–38].

Rf values obtained in toluene:ethyl acetate:formic acid (2:8:1, v/v/v) as the mobile phase (MPA) ranged from 0.017 to 0.756 with most of the standards presenting Rf values at around 0.600. Rf values obtained using the slightly lower polar mobile phase of toluene:ethyl acetate:formic acid (6:5:1, v/v/v) (MPB) ranged from 0.012 to 0.687 with most of the standards presenting Rf values at around 0.500.

6-Hydroxyflavone-β-D-glucoside (2), baicalin (5), vitexin (9), rutin (17), hesperidin (19), naringin (21), genistin (34), ellagic acid (42), leptospermine (46), chlorogenic acid (64) and neochlorogenic acid (69) were found to have relatively low Rf values in both solvent systems with Rf values ranging from 0.017 to 0.211 and 0.012 to 0.072 for MPA and MPB, respectively. This most likely reflects the presence of esters of sugar moieties or quinic acid (except for ellagic acid). The Rf values obtained using MPA were utilised in the development of databases 1A and 1B, while the Rf values obtained using MPB were used to establish databases 2A and 2B. To account for potential, slight, inter-run variations in the Rf values, the filtering threshold for the Rf values was set at ±0.05.
2.1.2. Colour

The colour of the standards after development and derivatisation were determined as these were found to be very important tools in discriminating between different compounds. Colours of the samples analysed were determined as fluorescence after being irradiated with UV light at 254 nm after development, UV light at 366 nm after development and also after derivatisation, and with white light in transmittance mode after derivatisation. The VisionCat software of the HPTLC originally generated colours using the RGB colour space (see Table 1). These colours were in a next step converted into hue values for easier comparisons based on a single numerical value.

The hue values obtained at 254 nm after development ranged from 120° to 163.8°, thus from green (120.0° to 149.9°) to turquoise (150° to 179.9°) upon conversion into colour families with most of the standards presenting hue values at around 134° (green). Only four standards had hue values greater than 150°, which indicates little variation and thus discriminatory power of the colour at this imaging condition. The colours at 254 nm after development were obtained individually for each condition; they were, however, found to be very similar and therefore only one dataset is presented in Table 1.

The hue values obtained at 366 nm after development ranged from orange to violet (33.0° to 255.5°), with most standards having a hue value of around 180° (cyan blue). A greater variance in colour was observed at this imaging condition, and thus the obtained hue values at 366 nm after development were found to be helpful in discriminating between the different standards. Similar to the colours obtained at 254 nm after development, very similar values were recorded at 366 nm after development in all four conditions, and thus only one dataset is represented in Table 1.

The use of natural product reagent (also known as Naturstoff reagent or Neu’s reagent, diphenylborinic acid 2-aminoethyl ester (DPBA) and 2-aminoethyl diphenylborinate, CAS No. 524-95-8) is one of the most popular methods of investigating the fluorescence of flavonoids. It has been extensively used as a spray reagent for flavonoid detection in chromatography, exerting its activity through chelation or coordination/complexation, which can be captured as a green-to-yellow-to-orange fluorescence on excitation with UV or blue light [39]. Additional advantages for its use are minimal interference from other compounds [39], easy reagent preparation and convenient drying in warm air [40].

The hue values obtained at 366 nm after derivatising with NP-PEG reagent ranged from red to scarlet (24.3° to 346.6°), with most standards having a hue value of around 160° (turquoise). A great variation in colours was observed at this imaging condition and thus this derivatisation method was found to be very helpful in discriminating compounds from each other. Very similar hue values were observed when the standards were derivatised with NP-PEG reagent regardless of the solvent system used; therefore, only one dataset was used to develop databases 1A and 2A (Table 1).

Vanillin reagent is a very popular spraying reagent in HPTLC analysis. It is used to detect terpenoids, sterols, salicin, ergot, alkaloids and most lipophilic compounds [41].

Hue values obtained at 366 nm after derivatising with VS reagent ranged from 178.5° to 339.5° (turquoise to scarlet), with most compounds having hues of around 215° (blue). Flavonoids were observed to have hue values ranging from 178.5° to 230.6° (turquoise to blue), hydroxybenzoic acid and its derivatives to have hues ranging from 199.4° to 253.3° (cyan blue to violet), whereas hydroxycinnamic acid and its derivatives presented hue values between 201.9° and 245.2° (cyan blue to violet)

When analysed with white light in transmittance mode, the hue values obtained after derivatisation with VSA reagent ranged from 0.00° to 356.0° (red to scarlet), with most of the compounds having hue values at around 41° (orange). Flavonoids were found to have hue values ranging from 327° to 54.2° (red to orange), hydroxybenzoic acid and its derivatives from 340.0° to 111.1° (magenta to yellow green) and hydroxycinnamic acid and its derivatives from 9.5° to 136.1° (red to green).
Very similar hue values were observed when the standards were derivatised with VSA reagent regardless of the solvent system used; therefore, only one dataset was used to develop databases 1B and 2B (Table 1).

To account for the potential slight inter-run variations in colour prior to and also after derivatisation, the filtering threshold for hue values was set at ±60°.

### 2.1.3. Fluorescence Spectra

The fluorescence spectra of the various standard compounds were obtained by scanning the standards from 190 nm to 390 nm. The spectra were carefully studied and the number of peaks as well as the respective \( \lambda \) max and \( \lambda \) min values were tabulated. Most standards had \( \lambda \) max values ranging between 220 and 270 nm.

\( \lambda \) max values obtained for repeat scans of each standard showed variations that were within ±2%; therefore, the \( \lambda \) max values for the different standards were considered as a reliable parameter in the establishment of all four databases (Table 1).

Similar to UV-Vis absorption behaviour, fluorescence is unique for each compound and can thus be used for the confirmation of chemical identity. For this study, the threshold for the database filtering was set at \( \lambda \) max ± 15 nm. Furthermore, the fluorescence spectra obtained for each standard were extracted as CSV files and used for spectral overlays in cases where the standard was considered as a potential candidate for matching with a band in the unknown.

### 2.1.4. UV-Vis Spectra

The UV-Vis spectra of compounds were obtained by scanning the standards from 190 nm to 900 nm. However, given the potential interferences from the mobile-phase solvents used, only absorbances between 250 nm and 500 nm were taken into account prior to and also after derivatisation with NP-PEG reagent, whereas absorbances ranging from 250 nm to 600 nm were considered after derivatisation with VSA reagent. The number of peaks of the spectra were identified and the \( \lambda \) max of each peak tabulated.

Most standards (73) presented 1 peak, although 2 peaks could be identified for 30 standards, while only 4 standards had 3 peaks within the examined region. Almost all standards presented maxima between 251 and 393 nm. The \( \lambda \) max values obtained on repeat scans of each standard showed variations that were within ±2%; therefore, the use of \( \lambda \) max values of the standards was considered as a reliable parameter in the establishment of all four databases (Table 1).

UV-Vis absorption behaviour is directly related to the structure of each compound, and its \( \lambda \) max values can therefore be used as a filtering criterion in compound matching. The thresholds for database filtering for \( \lambda \) max values of the UV-Vis spectra were set at ±15 nm before derivatisation and ±60 nm after derivatisation. Furthermore, the spectra obtained for each standard were extracted as CSV files and used for spectral overlays in cases where the standard was considered a potential candidate for matching with a band in the unknown sample.

### 2.1.5. Data Filtering

In order to try and match a band in the unknown sample with a standard in the database, the following filtering approach was adopted: the Rf value ±0.05 was set as the primary filtering parameter, followed by screening based on colour hue (±60°). The reduced list of potential candidates was filtered further using the fluorescence \( \lambda \) max (±15) and \( \lambda \) min (±15) values prior to derivatisation, \( \lambda \) max values (±15) of the UV-Vis spectrum prior to derivatisation, number of peaks of the UV-Vis spectrum prior to derivatisation, \( \lambda \) max values (±15) of the fluorescence spectrum after derivatisation and, finally, \( \lambda \) max values (±60) of the UV-Vis spectrum after derivatisation. The next step was the spectral overlays of the UV-Vis prior to and after derivatisation between potential matches and the unknown compound. A compound was considered a candidate match if it met all the
criteria enumerated above, and if its spectral overlays showed a substantial similarity both qualitatively and quantitatively. The same approach was adopted for all four databases.

2.2. Validation of Databases Using Spiked Artificial Honey

Three test compounds were individually spiked into artificial honey to validate the filtering approach used in the application of the database. Test compounds A and C were hydroxybenzoic acid derivatives and test compound B a flavonoid. Both test compounds A and B were the standards in the database (positive control), whereas test compound C was not included (negative control).

The results of the database validation using these test compounds are detailed in Tables 2–15. In all of these tables, the first row shows the Rf and hue of the respective test compound as well as the λmax and min of the fluorescence spectra prior to derivatisation, UV-Vis λmax prior to derivatisation, and fluorescence λmax and UV-Vis λmax after derivatisation with either of the two derivatising agents. The second row summarises the number of potential hits employing each filtering criterion, and the rows below list the potential hits and their respective data. As subsequent filtering criterions were applied, the number of potential hits reduced.

Table 2. Result of compound matching for test compound A using DB 1A.

| Name and Code | Rf | H' DEV 254 nm | H' DEV 366 nm | H' NP 366 nm | FL DEV λ | FL DEV λ | UV DEV λ | UV DEV λ | UV DEV λ | UV DEV λ | UV DEV λ | UV DEV λ |
|---------------|----|---------------|---------------|---------------|------------|------------|----------|----------|----------|----------|----------|----------|
| Test Compound A | 0.608 | 139 | 180 | 209 | 225 | 258 | 276 | 0 | 0 | 239 | 288 | 0 | 0 |
| 3,4,5-THBA, (36) | 0.589 | 138 | 185 | 216 | 223 | 256 | 261 | 0 | 0 | 236 | 306 | 0 | 0 |
| Eudesmic acid, (43) | 0.617 | 139 | 180 | 203 | 228 | 254 | 294 | 0 | 0 | 232 | 291 | 0 | 0 |
| Methyl Syringate, (48) | 0.610 | 138 | 175 | 203 | 237 | 258 | 277 | 0 | 0 | 241 | 291 | 0 | 0 |
| Syringic Acid, (58) | 0.577 | 137 | 192 | 215 | 225 | 258 | 277 | 0 | 0 | 240 | 290 | 0 | 0 |
| M-Coumaric Acid, (67) | 0.633 | 143 | 215 | 220 | 224 | 252 | 280 | 0 | 0 | 228 | 288 | 0 | 0 |
| Abscisic Acid, (103) | 0.598 | 127 | 171 | 205 | 221 | 251 | 268 | 0 | 0 | 225 | 295 | 0 | 0 |
| Isorhamnetin, (12) | 0.651 | 136 | 180 | 164 | 224 | 255 | 261 | 0 | 0 | 223 | 274 | 0 | 0 |
| Kaempferol, (14) | 0.654 | 131 | 150 | 150 | 223 | 254 | 269 | 0 | 0 | 222 | 288 | 0 | 0 |
| Protocatechuic Acid, (55) | 0.577 | 136 | 183 | 246 | 225 | 255 | 261 | 0 | 0 | 223 | 275 | 0 | 0 |
| Vanillic Acid, (59) | 0.623 | 136 | 192 | 209 | 228 | 255 | 263 | 0 | 0 | 225 | 275 | 0 | 0 |
| 3,5-DHBA, (39) | 0.594 | 138 | 191 | 217 | 225 | 251 | 0 | 0 | 227 | 288 | 0 | 0 |
| Gentisic Acid, (45) | 0.626 | 143 | 224 | 246 | 224 | 246 | 0 | 0 | 225 | 275 | 0 | 0 |
| m-HBA, (50) | 0.644 | 138 | 181 | 184 | 223 | 245 | 0 | 0 | 224 | 275 | 0 | 0 |
| o-Anisic Acid, (52) | 0.589 | 138 | 206 | 193 | 221 | 243 | 0 | 0 | 225 | 252 | 0 | 0 |
| p-HBA, (54) | 0.637 | 130 | 184 | 180 | 225 | 252 | 0 | 0 | 224 | 249 | 0 | 0 |
| Resorcylic Acid, (56) | 0.630 | 144 | 214 | 226 | 224 | 249 | 0 | 0 | 224 | 250 | 0 | 0 |
| Caffeic Acid, (61) | 0.589 | 139 | 211 | 197 | 224 | 250 | 0 | 0 | 223 | 247 | 0 | 0 |
| CADE, (62) | 0.609 | 142 | 239 | 223 | 223 | 247 | 0 | 0 | 223 | 246 | 0 | 0 |
| Ferulic Acid, (65) | 0.616 | 139 | 209 | 206 | 224 | 246 | 0 | 0 | 224 | 246 | 0 | 0 |
| Isoferulic Acid, (66) | 0.600 | 140 | 227 | 216 | 224 | 248 | 0 | 0 | 222 | 248 | 0 | 0 |
| p-Coumaric Acid, (71) | 0.630 | 136 | 199 | 205 | 223 | 258 | 0 | 0 | 222 | 258 | 0 | 0 |
| Daidzein, (31) | 0.600 | 137 | 161 | 163 | 222 | 252 | 0 | 0 | 223 | 250 | 0 | 0 |
| Formononetin, (32) | 0.637 | 147 | 195 | 194 | 223 | 250 | 0 | 0 | 222 | 245 | 0 | 0 |
| Sinapic Acid, (74) | 0.630 | 139 | 224 | 198 | 222 | 245 | 0 | 0 | 222 | 245 | 0 | 0 |
| HVA, (79) | 0.654 | 137 | 177 | 213 | 222 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| p-HPAA, (82) | 0.620 | 138 | 179 | 180 | 222 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
Table 2. Cont.

| Name and Code | Rf 1 | H° DEV 254 nm | H° DEV 366 nm | H° NP 366 nm | FID Δλ | F1 Δλ m | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | Fl NP | UV NP λ1 | UV NP λ2 | UV NP λ3 |
|---------------|------|---------------|---------------|---------------|--------|--------|-----------|-----------|-----------|-------|----------|----------|----------|
| Test Compound A | 0.608 | 139 | 180 | 209 | 225 | 258 | 276 | 0 | 0 | 239 | 288 | 0 | 0 |
| Number of Potential Matches | 42 | 42 | 40 | 31 | 31 | 25 | 11 | 6 | 6 | 5 | 5 | 5 | 5 |
| 2,3,4-THBA, (36) | 0.589 | 138 | 185 | 216 | 223 | 256 | 267 | 0 | 0 | 236 | 280 | 0 | 0 |
| Eudesmic acid, (43) | 0.617 | 139 | 180 | 203 | 228 | 254 | 264 | 0 | 0 | 232 | 291 | 0 | 0 |
| Methyl Syringate, (48) | 0.610 | 138 | 175 | 203 | 237 | 258 | 277 | 0 | 0 | 241 | 291 | 0 | 0 |
| Syringic Acid, (58) | 0.577 | 137 | 152 | 215 | 225 | 258 | 277 | 0 | 0 | 240 | 290 | 0 | 0 |
| m-Coumaric Acid, (67) | 0.633 | 143 | 215 | 220 | 224 | 252 | 280 | 0 | 0 | 228 | 288 | 0 | 0 |
| Abscisic Acid, (103) | 0.598 | 127 | 171 | 205 | 221 | 251 | 268 | 0 | 0 | 228 | 288 | 0 | 0 |
| isorhamnetin, (12) | 0.651 | 136 | 180 | 167 | 224 | 255 | 261 |
| Kaempferol, (14) | 0.654 | 131 | 150 | 150 | 223 | 254 | 269 |
| Protocatechuic Acid, (35) | 0.577 | 136 | 183 | 216 | 225 | 255 | 261 |
| Vanillic Acid, (59) | 0.623 | 136 | 192 | 209 | 228 | 255 | 263 |
| o-Coumaric Acid, (70) | 0.646 | 146 | 205 | 204 | 224 | 258 | 279 |
| 3,5-DHBA, (39) | 0.594 | 138 | 191 | 217 | 225 | 251 |
| Gentisic Acid, (45) | 0.626 | 143 | 224 | 245 | 224 | 246 |
| m-HBA, (50) | 0.644 | 138 | 181 | 184 | 223 | 245 |
| o-Anisic Acid, (52) | 0.589 | 138 | 206 | 193 | 221 | 243 |
| p-HBA, (54) | 0.637 | 130 | 184 | 180 | 225 | 252 |
| Resorcylic Acid, (56) | 0.630 | 144 | 214 | 226 | 224 | 249 |
| Caffeic Acid, (61) | 0.589 | 139 | 211 | 197 | 224 | 250 |
| CADE, (62) | 0.609 | 142 | 223 | 223 | 223 | 247 |
| Ferulic Acid, (65) | 0.616 | 139 | 209 | 206 | 224 | 246 |
| Isoflavone Acid, (66) | 0.600 | 140 | 227 | 216 | 224 | 248 |
| p-Coumaric Acid, (71) | 0.630 | 136 | 199 | 205 | 223 | 258 |
| Daidzein, (31) | 0.600 | 137 | 161 | 203 | 222 | 251 |
| Formononetin, (32) | 0.637 | 147 | 195 | 194 | 223 | 250 |
| Sinapic Acid, (74) | 0.630 | 139 | 224 | 198 | 222 | 245 |
| HVA, (79) | 0.654 | 137 | 177 | 213 | 222 |
| p-HPAA, (82) | 0.620 | 138 | 179 | 180 | 222 |
| DL-DL-PLA, (84) | 0.616 | 131 | 120 | 171 | 223 |
| Phloretic Acid, (87) | 0.630 | 141 | 188 | 190 | 221 |
| ProCatd, (98) | 0.594 | 135 | 232 | 233 | 221 |
| Vanillin, (99) | 0.637 | 136 | 222 | 224 | 221 |
| Acacetin, (3) | 0.649 | 135 | 180 |
| Apigenin, (4) | 0.617 | 134 | 181 |
| Myricetin, (15) | 0.587 | 130 | 132 |
| Quercetin, (16) | 0.617 | 126 | 127 |
| Taxifolin, (25) | 0.591 | 135 | 183 |
| 2,3,4-TMBA, (37) | 0.630 | 131 | 175 |
| p-MCA, (72) | 0.650 | 137 | 191 |
| Rosmarinic Acid, (73) | 0.630 | 131 | 175 |
| Pyrogallol, (94) | 0.602 | 137 | 186 |
| Genkwanin, (7) | 0.653 | 139 |
| Luteolin, (8) | 0.584 | 129 |

Legend: Rf1—retention factor in MPA, H° DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H° DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H° NP 366 nm—hue equivalent at 366 nm after derivatisation with NP-PEG-derivatisation reagent, Fl DEV λ—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ1—UV-Vis λ max prior to derivatisation, Fl NP λ—fluorescence λ max after derivatisation with NP-PEG reagent, UV NP λ1—UV-Vis λ max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.
Table 3. Result of compound matching for test compound A using DB 1B.

| Name and Code               | Rf 1 | H$^+$ DEV 254 nm | H$^+$ DEV 366 nm | H$^-$ VS 366 nm | H$^+$ TVSA | FID $\lambda_1$ | FID $\lambda_2$ | FID $\lambda_3$ | UV $\lambda_1$ | UV $\lambda_2$ | UV $\lambda_3$ | Fl VS $\lambda$ | Fl UV $\lambda$ |
|-----------------------------|------|------------------|------------------|-----------------|-------------|----------------|----------------|----------------|---------------|---------------|---------------|----------------|----------------|
| **Test Compound A**         | 0.580| 137              | 180              | 204             | 11           | 224           | 257           | 277            | 0             | 0             | 251           | 354            |
| **Number of Potential Matches** | 32       | 32               | 32               | 29              | 27           | 27            | 22            | 8              | 5             | 5             | 5             | 2              |
| Methyl Syringate, (48)      | 0.610| 138              | 175              | 219             | 13           | 237           | 258           | 277            | 0             | 0             | 248           | 351            |
| Syringic Acid, (58)         | 0.577| 137              | 192              | 218             | 23           | 237           | 258           | 277            | 0             | 0             | 248           | 351            |
| 2,3,4-THBA, (36)            | 0.589| 138              | 185              | 226             | 21           | 223           | 256           | 267            | 0             | 0             | 248           | 351            |
| Eudesmic Acid, (43)         | 0.617| 139              | 180              | 219             | 17           | 228           | 254           | 264            | 0             | 0             | 248           | 351            |
| Apigenin, (4)               | 0.617| 134              | 199              | 206             | 46           | 224           | 256           | 271            |               |               |               |                 |
| Fisetin, (10)               | 0.540| 138              | 176              | 184             | 52           | 224           | 254           | 264            |               |               |               |                 |
| Vanillic Acid, (59)         | 0.623| 136              | 192              | 218             | 32           | 228           | 255           | 263            |               |               |               |                 |
| Myricetin, (15)             | 0.587| 130              | 132              | 189             | 40           | 224           | 253            |               |               |               |               |                 |
| Quercetin, (16)             | 0.617| 126              | 127              | 192             | 41           | 224           | 254            |               |               |               |               |                 |
| Daidzein, (31)              | 0.600| 137              | 161              | 226             | 27           | 222           | 251            |               |               |               |               |                 |
| 2,3,4-TMBA, (37)            | 0.602| 142              | 120              | 241             | 22           | 225           | 254            |               |               |               |               |                 |
| 3,5-DHBA, (39)              | 0.594| 138              | 191              | 222             | 33           | 225           | 251            |               |               |               |               |                 |
| Gentisic Acid, (45)         | 0.626| 143              | 224              | 213             | 13           | 224           | 246            |               |               |               |               |                 |
| o-Anisic Acid, (52)         | 0.589| 138              | 206              | 225             | 11           | 221           | 243            |               |               |               |               |                 |
| Protocatechuic Acid, (55)   | 0.577| 136              | 183              | 214             | 46           | 225           | 255            |               |               |               |               |                 |
| Resorcylic Acid, (56)       | 0.630| 144              | 214              | 202             | 57           | 224           | 249            |               |               |               |               |                 |
| Caffeic Acid, (61)          | 0.589| 139              | 211              | 215             | 38           | 224           | 250            |               |               |               |               |                 |
| Ferulic Acid, (65)          | 0.616| 139              | 209              | 215             | 41           | 224           | 246            |               |               |               |               |                 |
| p-Coumaric Acid, (71)       | 0.630| 136              | 199              | 207             | 43           | 223           | 258            |               |               |               |               |                 |
| Rosmarinic Acid, (73)       | 0.630| 131              | 175              | 226             | 44           | 224           | 248            |               |               |               |               |                 |
| Sinapic Acid, (74)          | 0.630| 139              | 224              | 224             | 46           | 222           | 243            |               |               |               |               |                 |
| Taxifolin, (25)             | 0.591| 135              | 183              | 206             | 24           | 224            |               |               |               |               |               |                 |
| HGA, (78)                   | 0.545| 139              | 184              | 237             | 49           | 224            |               |               |               |               |               |                 |
| DL-p-HPLA, (84)             | 0.616| 139              | 120              | 244             | 44           | 223            |               |               |               |               |               |                 |
| Phloretic Acid, (87)        | 0.630| 141              | 188              | 229             | 22           | 221            |               |               |               |               |               |                 |
| Procatal, (98)              | 0.594| 135              | 232              | 240             | 32           | 224            |               |               |               |               |               |                 |
| CADE, (62)                  | 0.609| 142              | 239              | 212             |               | 221            |               |               |               |               |               |                 |
| Isoferulic Acid, (66)       | 0.600| 140              | 227              | 215             |               | 224            |               |               |               |               |               |                 |
| 3,4-DHPAA, (77)             | 0.556| 137              | 180             |               |               | 224            |               |               |               |               |               |                 |
| p-HPAA, (82)                | 0.620| 138              | 179             |               |               | 224            |               |               |               |               |               |                 |
| Abscisic Acid, (103)        | 0.598| 127              | 171            |               |               | 224            |               |               |               |               |               |                 |
| Luteolin, (8)               | 0.584| 129              |               |               |               | 224            |               |               |               |               |               |                 |

Legend: Rf1—retention factor in MPA, H$^+$ DEV 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H$^+$ DEV 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H$^-$ VS 366 nm—hue and colour equivalent at 366 nm after derivatisation w/ VSA-derivatisation reagent, H$^+$ TVSA—hue and colour equivalent at transmittance in white light after derivatisation w/ VSA-derivatisation reagent; Fl DEV $\lambda_{max}$—fluorescence $\lambda_{max}$ prior to derivatisation, Fl DEV $\lambda_{min}$—fluorescence $\lambda_{min}$ prior to derivatisation, UV DEV $\lambda_{1,3}$—UV-Vis $\lambda_{max}$ prior to derivatisation, Fl VS $\lambda$—fluorescence $\lambda$ max after derivatisation with VSA reagent, UV-Vis $\lambda$—UV-Vis $\lambda_{max}$ max after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.
Table 4. Results of compound matching for test compound A using DB 2A.

| Name and Code | Rf 2 | H° DEV 254 nm | H° DEV 366 nm | H° NP 366 nm | Fl DEV λ | Fl DEV λm | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | Fl NP λ | UV NP λ1 | UV NP λ2 |
|--------------|------|---------------|---------------|--------------|-----------|-----------|----------|----------|----------|----------|---------|--------|
| Test Compound A | 0.435 | 139 | 180 | 204 | 225 | 258 | 276 | 0 | 0 | 239 | 288 | 0 |
| Number of Potential Matches | 18 | 18 | 17 | 15 | 15 | 10 | 5 | 3 | 3 | 2 | 2 | 2 |
| 2,3,4-THBA, (36) | 0.437 | 138 | 185 | 216 | 223 | 256 | 267 | 0 | 0 | 236 | 306 | 0 |
| Syringic Acid, (58) | 0.444 | 137 | 192 | 215 | 225 | 258 | 277 | 0 | 0 | 240 | 290 | 0 |
| Abscisic Acid, (103) | 0.435 | 127 | 171 | 205 | 221 | 251 | 268 | 0 | 0 | 215 | 268 | 0 |
| 5-Methoxyflavone, (1) | 0.422 | 164 | 221 | 234 | 225 | 254 | 268 | 0 | 0 | 225 | 255 | 261 |
| Protocatechuic Acid, (55) | 0.398 | 136 | 183 | 246 | 225 | 255 | 268 | 0 | 0 | 225 | 255 | 261 |
| 3,5-DHBA, HBAD, (39) | 0.428 | 138 | 191 | 217 | 225 | 251 | 251 | 0 | 0 | 251 | 251 | 251 |
| Caffeic Acid, (61) | 0.421 | 139 | 211 | 197 | 224 | 250 | 250 | 0 | 0 | 250 | 250 | 250 |
| Isoferulic Acid, (66) | 0.459 | 140 | 227 | 216 | 224 | 248 | 248 | 0 | 0 | 248 | 248 | 248 |
| Sinapic Acid, (74) | 0.439 | 139 | 224 | 198 | 222 | 245 | 245 | 0 | 0 | 245 | 245 | 245 |
| Daidzein, (31) | 0.422 | 137 | 161 | 226 | 222 | 251 | 251 | 0 | 0 | 251 | 251 | 251 |
| HVA, (79) | 0.441 | 137 | 177 | 213 | 222 | 252 | 252 | 0 | 0 | 252 | 252 | 252 |
| p-HFPA, (82) | 0.471 | 138 | 179 | 180 | 222 | 252 | 252 | 0 | 0 | 252 | 252 | 252 |
| DL-p-HPLA, (84) | 0.466 | 139 | 120 | 171 | 223 | 253 | 253 | 0 | 0 | 253 | 253 | 253 |
| Phlorotic Acid, (87) | 0.483 | 141 | 188 | 190 | 221 | 254 | 254 | 0 | 0 | 254 | 254 | 254 |
| Procata, (98) | 0.453 | 135 | 232 | 233 | 221 | 254 | 254 | 0 | 0 | 254 | 254 | 254 |
| 2,3,4-TMBA, (37) | 0.479 | 142 | 120 | 120 | 221 | 254 | 254 | 0 | 0 | 254 | 254 | 254 |
| Pyrogallol, (94) | 0.445 | 137 | 186 | 186 | 221 | 254 | 254 | 0 | 0 | 254 | 254 | 254 |
| Luteolin, (8) | 0.418 | 129 | 187 | 187 | 221 | 254 | 254 | 0 | 0 | 254 | 254 | 254 |

Legend: Rf2—retention factor in MPB, H° DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H° DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H° NP 366 nm—hue equivalent at 366 nm after derivatisation w/ NP-PEG-derivatisation reagent, Fl DEV λ—fluorescence λ max prior to derivatisation, Fl DEV λm—fluorescence λ min prior to derivatisation, UV DEV λ1-3—UV-Vis λ max prior to derivatisation, FI NP λ—fluorescence λ max after derivatisation with NP-PEG reagent, UV NP λ1-3—UV-Vis λ max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.

2.2.1. Test Compound A

As demonstrated in Table 2, the Rf value of test compound A in MBA (databases 1A and 1B) is observed to be 0.608, and by applying the ±0.05 filtering criterion, 42 standards remain as potential candidates. The hue (H°) value of test compound A at 254 nm after development was 139.3°. By applying ±60° as the filtering criterion, the number of potential matches in the database remained at 42. At 366 nm after development, the H° of test compound A was found to be 180°, which reduced the number of potential matches in the database to 40 based on the established H° ±60° filtering approach. After derivatisation with NP-PEG, the hue of test compound A was found to be 209.2°. By applying the ±60° filter criterion, the remaining number of potential matches in the database was 31. The fluorescence λ max of unknown A was found to be 225 nm. Using the ±15 nm filtering range, the number of matches remained at 31. However, when the λ min (at 258 nm) of the spectra was used and the filter applied, the number of potential matches was reduced to 25. The first UV-Vis λ max of test compound A was detected at 276 nm. Upon using the ±15 nm filtering range, the number of potential matches was narrowed to 11. As test compound A only had 1 λ max value, potential matches with a total of 2 or 3 maxima could be eliminated reducing the number of potential matches to 6. After derivatisation with NP-PEG, the fluorescence λ max of unknown A was found to be 239 nm, and by using the ±15 nm filtering range the number of potential matches was reduced further to 5. When considering the UV-Vis λ max after derivatisation with NP-PEG (288 nm) and applying the screening criterion of ±60 nm, the number of potential matches remained at 5. Thus, by applying the filtering approach outlined in Section 2.1.5, 5 compounds were considered.
as potential matches against database 1A, namely, 2,3,4-trihydroxybenzoic acid, eudesmic acid, methyl syringate, syringic acid and m-coumaric acid.

Table 5. Results of compound matching for test compound A using DB 2B.

| Name and Code | Rf 2 | H° DEV 254 nm | H° DEV 366 nm | H° VSA 366 nm | H° T VSA | FI DEV λ | FI DEV λ m | UV DEV λ 1 | UV DEV λ 2 | UV DEV λ 3 | FI VS λ | UV VS λ |
|---------------|------|---------------|---------------|---------------|----------|-----------|------------|-----------|-----------|-----------|-----------|---------|
| Test Compound A | 0.435 | 137 | 180 | 204 | 14 | 224 | 257 | 277 | 0 | 0 | 251 | 354 |
| Number of Potential Matches | 18 | 18 | 17 | 15 | 14 | 10 | 4 | 3 | 3 | 3 | 1 |
| Syringic Acid, (58) | 0.444 | 137 | 192 | 218 | 28 | 225 | 258 | 277 | 0 | 0 | 247 | 351 |
| 2,3,4-THBA, (36) | 0.437 | 138 | 185 | 226 | 21 | 223 | 256 | 267 | 0 | 0 | 248 |
| Pyrogallol, (94) | 0.445 | 137 | 186 | 224 | 21 | 224 | 255 | 270 | 0 | 0 | 263 |
| 5-Methoxyflavone, (1) | 0.422 | 164 | 221 | 187 | 35 | 225 | 254 | 268 |
| Daidzein, (31) | 0.422 | 137 | 161 | 226 | 29 | 222 | 251 |
| 3,5-DHBA, (39) | 0.428 | 138 | 191 | 222 | 33 | 225 | 251 |
| Protocatechuic Acid, (55) | 0.398 | 136 | 183 | 214 | 46 | 225 | 255 |
| Caffeic Acid, (61) | 0.421 | 139 | 211 | 215 | 38 | 224 | 250 |
| Sinapic Acid, (74) | 0.439 | 139 | 224 | 224 | 46 | 222 | 245 |
| Homovanillic Acid, (79) | 0.441 | 137 | 177 | 230 | 67 | 222 |
| DL-p-HPLA, (84) | 0.466 | 139 | 120 | 241 | 22 | 225 | 254 |
| Phloretic Acid, (87) | 0.483 | 141 | 188 | 229 | 33 | 221 |
| Protocatechualdehyde, (98) | 0.453 | 135 | 232 | 240 | 29 | 221 |
| Isoferulic Acid, (66) | 0.459 | 140 | 227 | 215 |
| p-HPAA, (82) | 0.471 | 138 | 179 |
| Absisic Acid, (103) | 0.435 | 127 | 171 |
| Luteolin, (8) | 0.418 | 129 |

Legend: Rf2—retention factor in MPB, H° DEV 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H° DEV 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H° VSA 366 nm—hue and colour equivalent at 366 nm after derivatisation with VSA-derivatisation reagent, H° T VSA—hue and colour equivalent at transmittance in white light after derivatisation with VSA derivatisation reagent, Fl DEV λ max—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ 1—UV-Vis λ max prior to derivatisation, FI NP λ—fluorescence λ max after derivatisation with VSA reagent, UV-Vis λ—UV-Vis λ max after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 6. Results of compound matching for test compound B using DB 1A.

| Name and Code | Rf 1 | H° DEV 254 nm | H° DEV 366 nm | H° NP 366 nm | Fl FI λ | Fl FI λ m | UV DEV λ 1 | UV DEV λ 2 | UV DEV λ 3 |
|---------------|------|---------------|---------------|---------------|--------|-----------|-----------|-----------|-----------|
| Test Compound B | 0.685 | 121 | 152 | 147 | 224 | 255 | 269 | 369 | 0 | 245 | 288 | 373 | 433 |
| Number of Potential Matches | 48 | 48 | 42 | 30 | 28 | 23 | 16 | 2 | 2 | 2 | 2 | 2 | 1 |
| Kaempferol, (14) | 0.654 | 131 | 150 | 180 | 223 | 254 | 269 | 371 | 0 | 245 | 291 | 369 | 432 |
| Isorhamnetin, (12) | 0.651 | 136 | 180 | 167 | 224 | 255 | 261 | 364 | 0 | 250 | 293 | 434 |
| Galangin, (11) | 0.702 | 131 | 172 | 172 | 224 | 251 | 268 |
| Kaempferide, (13) | 0.690 | 129 | 144 | 161 | 223 | 254 | 269 |
| Biochanin A, (30) | 0.692 | 133 | 97 | 156 | 224 | 252 | 262 |
| Genistein, (33) | 0.663 | 128 | 181 | 136 | 224 | 253 | 260 |
| Benzoic Acid, (40) | 0.663 | 136 | 185 | 159 | 211 | 241 | 276 |
Table 6. Cont.

| Name and Code          | Rf1 | H\(^{+}\) DEV 254 nm | H\(^{+}\) DEV 366 nm | H\(^{+}\) NP-366 nm | Fl DEV\(\lambda\) m | FI NP\(\lambda\) | UV DEV\(\lambda_1\) | UV DEV\(\lambda_2\) | UV DEV\(\lambda_3\) | UV NP\(\lambda_1\) | UV NP\(\lambda_2\) | UV NP\(\lambda_3\) |
|------------------------|-----|----------------------|----------------------|---------------------|-------------------|--------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Methyl-3,4,5-TMBz, (49)| 0.670| 141                  | 186                  | 120                 | 230               | 256          | 265              |                  |                  |                  |                  |                  |
| m-Toluic Acid, (51)    | 0.681| 137                  | 160                  | 189                 | 221               | 242          | 284              |                  |                  |                  |                  |                  |
| p-HBA, (54)            | 0.657| 130                  | 184                  | 180                 | 225               | 252          | 257              |                  |                  |                  |                  |                  |
| o-Coumaric Acid, (70)  | 0.646| 146                  | 205                  | 204                 | 224               | 258          | 279              |                  |                  |                  |                  |                  |
| 2-M-4-VPh, (88)        | 0.688| 122                  | 189                  | 191                 | 224               | 251          | 266              |                  |                  |                  |                  |                  |
| 4-MPCat, (90)          | 0.674| 137                  | 180                  | 161                 | 223               | 256          | 283              |                  |                  |                  |                  |                  |
| 2-MBzd, (96)           | 0.709| 134                  | 188                  | 194                 | 222               | 253          | 255              |                  |                  |                  |                  |                  |
| 2′-HAPh, (100)         | 0.704| 133                  | 152                  | 180                 | 228               | 254          | 256              |                  |                  |                  |                  |                  |
| Benzo phenone, (104)   | 0.727| 120                  | 171                  | 196                 | 224               | 253          | 260              |                  |                  |                  |                  |                  |
| Naringenin, (20)       | 0.680| 137                  | 177                  | 160                 | 223               | 246          |                  |                  |                  |                  |                  |                  |
| Pinocembrin, (22)      | 0.723| 137                  | 179                  | 160                 | 224               | 241          |                  |                  |                  |                  |                  |                  |
| Formononetin, (32)     | 0.637| 147                  | 195                  | 194                 | 223               | 250          |                  |                  |                  |                  |                  |                  |
| Cumicnic Acid, (41)    | 0.697| 136                  | 180                  | 185                 | 221               | 246          |                  |                  |                  |                  |                  |                  |
| m-HBA, (50)            | 0.644| 138                  | 181                  | 184                 | 223               | 245          |                  |                  |                  |                  |                  |                  |
| Salicylic Acid, (57)   | 0.679| 140                  | 211                  | 181                 | 225               | 242          |                  |                  |                  |                  |                  |                  |
| p-MCA, (72)            | 0.650| 137                  | 171                  | 160                 | 222               | 257          |                  |                  |                  |                  |                  |                  |
| Hesperetin, (18)       | 0.667| 137                  | 169                  | 160                 | 222               |              |                  |                  |                  |                  |                  |                  |
| Sakuranetin, (23)      | 0.699| 137                  | 160                  | 102                 | 223               |              |                  |                  |                  |                  |                  |                  |
| Pinobanksin, (24)      | 0.697| 137                  | 191                  | 174                 | 224               |              |                  |                  |                  |                  |                  |                  |
| Trans-p-CAME, (76)     | 0.674| 136                  | 193                  | 202                 | 222               |              |                  |                  |                  |                  |                  |                  |
| p-MPLA, (85)           | 0.658| 138                  | 179                  | 192                 | 222               |              |                  |                  |                  |                  |                  |                  |
| Isops, (91)            | 0.725| 138                  | 179                  | 190                 |                  |              |                  |                  |                  |                  |                  |                  |
| Thymol, (92)           | 0.732| 140                  | 120                  | 131                 |                  |              |                  |                  |                  |                  |                  |                  |
| Acacetin, (3)          | 0.649| 135                  | 180                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Chrysin, (6)           | 0.672| 132                  | 186                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| t-Chalcone, (35)       | 0.734| 137                  | 113                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Methyl Paraben, (47)   | 0.676| 128                  | 179                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| o-Toluic Acid, (53)    | 0.692| 138                  | 175                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| VAME, (60)             | 0.658| 135                  | 179                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| HVA, (79)              | 0.654| 137                  | 177                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Phenylacetic Acid, (81)| 0.670| 136                  | 175                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| 3-PAA, (86)            | 0.683| 139                  | 178                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| p-Methoxyphenol, (89)  | 0.685| 134                  | 171                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Pyrocatechol, (95)     | 0.662| 137                  | 181                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| p-Anisaldehyde, (97)   | 0.660| 120                  | 203                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Genkwatin, (7)         | 0.553| 139                  |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| CAPE, (63)             | 0.676| 140                  |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Methyl Ferulate, (68)  | 0.667| 138                  |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| t-Cinnamic Acid, (75)  | 0.672| 140.3                 |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| Vanillin, (99)         | 0.637| 136.2                 |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |
| 2′-MAPh, (101)         | 0.669| 139.1                 |                  |                  |                  |              |                  |                  |                  |                  |                  |                  |

Legend: Rf1—retention factor in MPA, H\(^{+}\) DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H\(^{+}\) DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H\(^{+}\) NP-366 nm—hue equivalent at 366 nm after derivatisation w/ NP-PEG-derivatisation reagent, Fl DEV\(\lambda\) m—fluorescence\(\lambda\) min prior to derivatisation, Fl NP\(\lambda\) m—fluorescence\(\lambda\) min after derivatisation, UV DEV\(\lambda_1,3\)—UV-Vis\(\lambda\) max prior to derivatisation, FI NP\(\lambda\) m—fluorescence\(\lambda\) max after derivatisation with NP-PEG reagent, UV NP\(\lambda_1,3\)—UV-Vis\(\lambda\) max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.
Table 7. Results of compound matching for test compound B using DB 1B.

| Name and Code | Rf1 | R' H' DEV 254 nm | H' DEV 366 nm | H' VSA 366 nm | H' T VSA | F DEV λ | F DEV λ | UV DEV λ | UV DEV λ | UV DEV λ | Fl DEV λ | UV VS λ | UV VS λ |
|----------------|-----|------------------|----------------|----------------|----------|---------|---------|----------|----------|----------|---------|---------|---------|
| Test Compound B | 0.650 | 137 | 174 | 190 | 36 | 223 | 256 | 269 | 371 | 0 | 251 | 377 |
| Number of Potential Matches | 58 | 58 | 51 | 48 | 46 | 45 | 34 | 22 | 3 | 3 | 3 | 3 |
|isorhamnetin, (12) | 0.651 | 136 | 180 | 191 | 43 | 224 | 255 | 261 | 364 | 223 | 254 | 269 | 371 | 0 | 250 | 380 |
|Kaempferol, (14) | 0.654 | 131 | 150 | 189 | 8 | 223 | 254 | 269 | 371 | 0 | 250 | 380 |
|Quercetin, (16) | 0.617 | 126 | 127 | 192 | 41 | 224 | 254 | 261 | 370 | 0 | 253 | 384 |
|Acacetin, (3) | 0.649 | 135 | 180 | 204 | 54 | 224 | 257 | 270 |
|Apigenin, (4) | 0.617 | 134 | 199 | 206 | 46 | 224 | 256 | 271 |
|Chrysin, (6) | 0.672 | 132 | 186 | 190 | 47 | 224 | 255 | 270 |
|Kaempferide, (13) | 0.690 | 129 | 144 | 188 | 45 | 223 | 254 | 269 |
|Genistein, (33) | 0.663 | 128 | 181 | 211 | 38 | 224 | 253 | 260 |
|2,3,4-TMBA, (37) | 0.602 | 142 | 120 | 241 |
|Benzoic Acid, (40) | 0.663 | 136 | 185 | 199 | 45 | 211 | 241 | 276 |
|Eudesmic Acid, (43) | 0.617 | 139 | 180 | 219 | 43 | 228 | 254 | 264 |
|Methyl Syringate, (48) | 0.610 | 138 | 175 | 219 | 43 | 237 | 258 | 277 |
|Methyl-3,4,5-TMBz, (49) | 0.670 | 141 | 186 | 202 | 42 | 230 | 256 | 265 |
|m-Ilosucle Acid, (51) | 0.681 | 137 | 160 | 206 | 32 | 221 | 242 | 284 |
|p-HBA, (54) | 0.637 | 130 | 184 | 212 | 26 | 225 | 252 | 257 |
|Vanillic Acid, (59) | 0.623 | 136 | 192 | 218 | 32 | 228 | 255 | 263 |
|VAME, (60) | 0.658 | 135 | 179 | 207 | 71 | 225 | 254 | 263 |
|m-Coumaric Acid, (67) | 0.633 | 143 | 215 | 208 | 49 | 224 | 252 | 280 |
|o-Coumaric Acid, (70) | 0.646 | 146 | 205 | 223 | 23 | 224 | 258 | 279 |
|4-MPCat, (90) | 0.674 | 137 | 180 | 242 | 245 | 223 | 256 | 283 |
|Pyrogallol, (94) | 0.662 | 137 | 181 | 219 | 36 | 226 | 265 | 277 |
|Naringenin, (20) | 0.68 | 137 | 177 | 211 | 24 | 223 | 246 |
|Formononetin, (32) | 0.637 | 147 | 195 | 179 | 35 | 223 | 250 |
|Cumic Acid, (41) | 0.697 | 136 | 180 | 205 | 2 | 221 | 246 |
|Gentisic Acid, (45) | 0.626 | 143 | 224 | 213 | 23 | 224 | 246 |
|m-HBA, (50) | 0.644 | 138 | 181 | 232 | 38 | 223 | 245 |
|Resorcylic Acid, (56) | 0.630 | 144 | 214 | 202 | 57 | 224 | 249 |
|Salicylic Acid, (57) | 0.679 | 140 | 211 | 205 | 48 | 223 | 242 |
|Ferulic Acid, (65) | 0.616 | 139 | 209 | 215 | 41 | 224 | 246 |
|p-Coumaric Acid, (71) | 0.630 | 136 | 199 | 207 | 41 | 223 | 258 |
|p-MCA, (72) | 0.650 | 137 | 191 | 245 | 10 | 222 | 257 |
|Rosmarinic Acid, (73) | 0.630 | 131 | 175 | 226 | 43 | 224 | 248 |
|Sinapic Acid, (74) | 0.630 | 139 | 224 | 224 | 46 | 224 | 245 |
|Hesperetin, (18) | 0.667 | 137 | 169 | 202 | 20 | 222 |
|Sakuranetin, (23) | 0.699 | 137 | 160 | 200 | 16 | 223 |
|Pinobanksin, (24) | 0.697 | 137 | 191 | 199 | 10 | 224 |
|o-Ilosucle Acid, (53) | 0.692 | 138 | 175 | 222 | 35 | 221 |
|trans-p-CAME, (76) | 0.674 | 136 | 193 | 205 | 49 | 222 |
|HVA, (79) | 0.654 | 137 | 177 | 230 | 32 | 222 |
|DL-p-PLA, (84) | 0.616 | 139 | 120 | 244 | 25 | 223 |
|p-MPLA, (85) | 0.658 | 138 | 179 | 228 | 33 | 222 |
|Phloretic Acid, (87) | 0.650 | 137 | 191 | 245 | 10 | 222 |
|p-Methoxyphenol, (89) | 0.685 | 134 | 171 | 227 | 37 | 223 |
|Vanillin, (99) | 0.637 | 136 | 222 | 212 | 42 | 221 |
|Phenylacetic Acid, (81) | 0.670 | 136 | 175 | 242 | 21 | 221 |
|Methyl Paraben, (47) | 0.676 | 128 | 179 | 228 |
|3-PPA, (86) | 0.683 | 139 | 178 | 231 |
|p-HIPAA, (82) | 0.620 | 138 | 179 |
|2-M-4-VPh, (88) | 0.688 | 122 | 189 |
Table 7. Cont.

| Name and Code              | RI1 | H° DEV 254 nm | H° DEV 366 nm | H° VSA 366 nm | H° T VSA | FIDEV λ  | Fl DEV λ max | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | UV VSA  | UV VSA  |
|----------------------------|-----|---------------|---------------|---------------|---------|----------|-------------|----------|----------|----------|---------|---------|
| p-Anisaldehyde, (97)      | 0.66| 120           | 203           |               |         |          |             |          |          |          |         |         |
| Genkwain, (7)             | 0.653| 139           |               |               |         |          |             |          |          |          |         |         |
| Biochanin A, (30)         | 0.692| 133           |               |               |         |          |             |          |          |          |         |         |
| CADE, (62)                | 0.609| 142           |               |               |         |          |             |          |          |          |         |         |
| CAPE, (63)                | 0.676| 140           |               |               |         |          |             |          |          |          |         |         |
| Methyl Ferulate, (68)     | 0.667| 138           |               |               |         |          |             |          |          |          |         |         |
| t-Cinnamic Acid, (75)     | 0.672| 140           |               |               |         |          |             |          |          |          |         |         |
| 2′-MAPh, (101)            | 0.669| 139           |               |               |         |          |             |          |          |          |         |         |

Legend: Rf2—retention factor in MPB, H° DEV 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H° DEV 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H° VS 366 nm—hue and colour equivalent at 366 nm after derivatisation w/ VSA-derivatisation reagent, H° T VS—hue and colour equivalent at transmittance in white light after derivatisation w/ VSA-derivatisation reagent, Fl DEV λ max—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ1,2,3—UV-Vis λ max prior to derivatisation, Fl VS λ—fluorescence λ max after derivatisation with VSA reagent, UV-Vis λ—UV-Vis λ max after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 8. Results of compound matching for test compound B using DB 2A.

| Name and Code              | RI2 | H° DEV 254 nm | H° DEV 366 nm | H° NP 366 nm | Fl DEV λ | Fl DEV λ m | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | UV VSA  | UV VSA  |
|----------------------------|-----|---------------|---------------|--------------|----------|------------|----------|----------|----------|---------|---------|
| Test Compound B            | 0.520| 135           | 152           | 147          | 224     | 255        | 269      | 369      | 0        | 245     | 433     | 288     | 373     |
| Number of Potential Matches| 33  | 135           | 152           | 147          | 224     | 255        | 269      | 369      | 0        | 245     | 432     | 291     | 369     |
| Kaempferol, (14)           | 0.547| 131           | 150           | 150          | 223     | 254        | 269      | 371      | 0        | 245     | 432     | 291     | 369     |
| Isorhamnetin, (12)         | 0.536| 136           | 180           | 167          | 224     | 255        | 261      | 364      | 0        | 250     | 434     | 293     |
| Apigenin, (4)              | 0.507| 134           | 199           | 109          | 224     | 256        | 271      |          |          |         |         |         |
| Genistein, (33)            | 0.549| 128           | 181           | 136          | 224     | 253        | 260      |          |          |         |         |         |
| 2,3,4-TMBA, (37)           | 0.479| 142           | 120           | 147          | 225     | 254        | 261      |          |          |         |         |         |
| Benzolic Acid, (40)        | 0.570| 136           | 185           | 159          | 211     | 241        | 276      |          |          |         |         |         |
| Eudesmic Acid, (43)        | 0.504| 139           | 180           | 203          | 228     | 254        | 264      |          |          |         |         |         |
| Methyl Syringate, (48)     | 0.524| 138           | 175           | 203          | 237     | 258        | 277      |          |          |         |         |         |
| p-Hydroxybenzoic Acid, (54)| 0.513| 130           | 184           | 180          | 225     | 252        | 257      |          |          |         |         |         |
| o-Coumaric Acid, (70)      | 0.519| 146           | 205           | 204          | 224     | 258        | 279      |          |          |         |         |         |
| 4-Methylpyrocatechol, (90) | 0.546| 147           | 180           | 161          | 223     | 256        | 283      |          |          |         |         |         |
| Formononetin, (32)         | 0.517| 147           | 195           | 194          | 223     | 250        |           |          |          |         |         |         |
| Cuminic Acid, (41)         | 0.525| 136           | 180           | 185          | 221     | 246        |           |          |          |         |         |         |
| m-Hydroxybenzoic Acid, (50)| 0.499| 138           | 181           | 184          | 223     | 245        |           |          |          |         |         |         |
| o-Anisic Acid, (52)        | 0.499| 138           | 206           | 193          | 221     | 243        |           |          |          |         |         |         |
| Ferulic Acid, (65)         | 0.508| 139           | 209           | 206          | 224     | 246        |           |          |          |         |         |         |
| p-Coumaric Acid, (71)      | 0.510| 136           | 199           | 205          | 223     | 258        |           |          |          |         |         |         |
| p-MCA, (72)                | 0.556| 137           | 191           | 131          | 222     | 257        |           |          |          |         |         |         |
| Hesperetin, (18)           | 0.559| 137           | 169           | 160          | 222     |           |          |          |          |         |         |         |
| p-IPAA, (82)               | 0.471| 138           | 179           | 180          | 222     |           |          |          |          |         |         |         |
| p-MPLA, (85)               | 0.536| 138           | 179           | 192          | 222     |           |          |          |          |         |         |         |
| Phloretic Acid, (87)       | 0.483| 141           | 188           | 190          | 221     |           |          |          |          |         |         |         |
| Acacetin, (3)              | 0.564| 135           | 180           |              |         |           |          |          |          |         |         |         |
| Vanillic Acid, (59)        | 0.504| 136           | 192           |              |         |           |          |          |          |         |         |         |
| VAME, (60)                 | 0.570| 135           | 179           |              |         |           |          |          |          |         |         |         |
| Pyrocatechol, (95)         | 0.554| 137           | 181           |              |         |           |          |          |          |         |         |         |
| Genkwain, (7)              | 0.533| 139           |               |              |         |           |          |          |          |         |         |         |
Table 8. Cont.

| Name and Code | RI2 | H’ DEV 254 nm | H’ DEV 366 nm | H’ NP 366 nm | Fl DEV λmin | Fl DEV λmax | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | Fl NP λmin | Fl NP λmax | UV NP λ1 | UV NP λ2 | UV NP λ3 |
|---------------|-----|---------------|---------------|--------------|-------------|-------------|------------|------------|------------|-------------|-------------|---------|---------|---------|
| Gentisic Acid, (45) | 0.508 | 143 | | | | | | | | | | | |
| Resorcylic Acid, (56) | 0.504 | 144 | | | | | | | | | | | |
| CADE, (62) | 0.501 | 142 | | | | | | | | | | | |
| CAPE, (63) | 0.504 | 140 | | | | | | | | | | | |
| m-Coumaric Acid, (67) | 0.493 | 143 | | | | | | | | | | | |
| Vanillin, (99) | 0.548 | 136 | | | | | | | | | | | |

Legend: RI2—retention factor in MPB, H’ DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H’ DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H’ NP 366 nm—hue equivalent at 366 nm after derivatisation w/ NP-PEG-derivatisation reagent, Fl DEV λ—fluorescence λ max prior to derivatisation, Fl DEV λ min—fluorescence λ min prior to derivatisation, UV DEV λ1,3—UV-Vis λ max prior to derivatisation, Fl NP λ—fluorescence λ max after derivatisation with NP-PEG reagent, UV NP λ1,3—UV-Vis λ max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 9. Results of compound matching for test compound B using DB 2B.

| Name and Code | RI2 | H’ DEV 254 nm | H’ DEV 366 nm | H’ NP 366 nm | Fl DEV λmin | Fl DEV λmax | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | Fl NP λmin | Fl NP λmax | UV NP λ1 | UV NP λ2 | UV NP λ3 |
|---------------|-----|---------------|---------------|--------------|-------------|-------------|------------|------------|------------|-------------|-------------|---------|---------|---------|
| Test Compound B | 0.52 | 137 | 174 | 190 | 36 | 223 | 256 | 269 | 371 | 0 | 251 | 377 |
| Number of Potential Matches | 34 | 34 | 31 | 30 | 30 | 30 | 26 | 16 | 2 | 2 | 2 | 2 |
| Isorhamnetin, (12) | 0.536 | 136 | 180 | 191 | 43 | 224 | 255 | 261 | 364 | 0 | 252 | 388 |
| Kaempferol, (14) | 0.547 | 131 | 150 | 189 | 8 | 223 | 254 | 269 | 371 | 0 | 250 | 380 |
| Acetin, (3) | 0.564 | 135 | 180 | 204 | 54 | 224 | 257 | 270 | | | | |
| Apigenin, (4) | 0.507 | 134 | 199 | 206 | 46 | 224 | 256 | 271 | | | | |
| Genistein, (33) | 0.549 | 128 | 181 | 211 | 34 | 224 | 253 | 260 | | | | |
| 2,3,4-TMBA, (37) | 0.479 | 142 | 120 | 241 | 42 | 225 | 254 | 261 | | | | |
| Benzoic Acid, (40) | 0.57 | 136 | 185 | 199 | 48 | 211 | 241 | 276 | | | | |
| Eudesmic Acid, (43) | 0.504 | 139 | 180 | 219 | 41 | 228 | 254 | 264 | | | | |
| Methyl Syringate, (48) | 0.524 | 138 | 175 | 219 | 43 | 237 | 258 | 277 | | | | |
| Vanillic Acid, (59) | 0.504 | 136 | 192 | 218 | 32 | 228 | 255 | 263 | | | | |
| VAME, (60) | 0.57 | 135 | 179 | 207 | 71 | 225 | 254 | 263 | | | | |
| m-Coumaric Acid, (67) | 0.493 | 143 | 215 | 208 | 46 | 224 | 252 | 280 | | | | |
| o-Coumaric Acid, (70) | 0.519 | 146 | 205 | 223 | 41 | 224 | 258 | 279 | | | | |
| 4-Methylpyrocatechol, (90) | 0.546 | 137 | 180 | 242 | 345 | 223 | 256 | 283 | | | | |
| Pyrocatechol, (95) | 0.554 | 137 | 181 | 219 | 36 | 226 | 265 | 277 | | | | |
| Dibenzyl Oxalate, (102) | 0.531 | 139 | 199 | 245 | 62 | 222 | 257 | 262 | | | | |
| Foromononetin, (32) | 0.517 | 147 | 195 | 179 | 38 | 223 | 250 | | | | | |
| Cuminc Acid, (41) | 0.525 | 136 | 180 | 205 | 2 | 221 | 246 | | | | | |
| Gentisic Acid, (45) | 0.508 | 143 | 224 | 213 | 49 | 224 | 246 | | | | | |
| m-HBA, (50) | 0.499 | 138 | 181 | 232 | 38 | 223 | 243 | | | | | |
| o-Anisic Acid, (52) | 0.499 | 138 | 206 | 225 | 43 | 221 | 243 | | | | | |
| p-HBA, (54) | 0.513 | 130 | 184 | 212 | 25 | 225 | 252 | | | | | |
| Resorcylic Acid, (56) | 0.504 | 144 | 214 | 202 | 57 | 224 | 249 | | | | | |
| Ferulic Acid, (65) | 0.508 | 139 | 209 | 215 | 41 | 224 | 246 | | | | | |
| p-Coumaric Acid, (71) | 0.51 | 136 | 199 | 207 | 41 | 223 | 258 | | | | | |
| p-MCA, (72) | 0.556 | 137 | 191 | 245 | 10 | 222 | 257 | | | | | |
| Hesperetin, (18) | 0.559 | 137 | 169 | 202 | 20 | 222 | | | | | | |
| p-MPLA, (85) | 0.536 | 138 | 179 | 228 | 38 | 222 | | | | | | |
| Phloretic Acid, (87) | 0.483 | 141 | 188 | 229 | 33 | 221 | | | | | | |
Table 9. Cont.

| Name and Code       | Rf/2  | H⁺ DEV 254 nm | H⁺ DEV 366 nm | H⁺ VSA 366 nm | H⁺ T VSA | FL DEV λ | FI DEV λ | UV DEV λ₁ | UV DEV λ₂ | UV DEV λ₃ | FI VS λ | UV VS λ |
|---------------------|-------|---------------|---------------|---------------|----------|-----------|----------|------------|------------|------------|----------|----------|
| Vanillin, (99)      | 0.548 | 136           | 222           | 212           | 212      | 0.767     | 0.942    | 0.976      | 0.976      | 0.976      | 100.0   | 100.0    |
| p-HPAA, (82)        | 0.471 | 138           | 179           | 179           |          |           |          |            |            |            |            |          |
| Genkwanin, (7)      | 0.533 | 139           | 179           | 179           | 179      |           |          |            |            |            | 100.0   |          |
| CAPE, (63)          | 0.501 | 142           | 190           |               |          |           |          |            |            |            |          |          |
|                    |       |               |               |               |          |           |          |            |            |            |          |          |

Legend: Rf2—retention factor in MPB, H⁺ DEV 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H⁺ DEV 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H⁺ VS 366 nm—hue and colour equivalent at 366 nm after derivatisation w/ VSA-derivatisation reagent, H⁺ T VS—hue and colour equivalent at transmittance in white light after derivatisation w/ VSA-derivatisation reagent; Fl DEV λ max—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ₁—UV-Vis λ max prior to derivatisation, UV-Vis λ—UV-Vis λ max after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 10. Summary of the correlations and % spectral matches used to determine the identity of test compound B.

| UNK DB | Rf  | Name and Code       | Rf  | UV DEV | % | UV NP | % | UV VS | % | Match       |
|--------|-----|---------------------|-----|--------|----|-------|----|-------|----|-------------|
| B      |     | Isorhamnetin, Flavonol (12) | 0.651 | 0.988 | 82.9 | 0.941 | 72.0 | 0.986 | 94.7 | Kaempferol   |
|        |     | Kaempferol, Flavonol (14) | 0.654 | 0.996 | 100.0 | 100.0 | 0.997 | 100.0 |         |             |
|        |     | Hesperetin, Flavanone (18) | 0.667 | 0.251 | 5.5  | 0.076 | 31.0 | 0.946 | 79.7 |             |
|        |     | Naringenin, Flavanone (20) | 0.680 | 0.229 | 6.6  | 0.281 | 27.8 | 0.203 | 13.9 |             |
|        |     | Benzoic Acid, HBAD (40)   | 0.663 | 0.922 | 41.8 | 0.294 | 18.3 | −0.08 | 59.1 |             |
|        |     | m-Toluic Acid, HBAD (51)  | 0.681 | 0.920 | 42.9 | −0.09 | 27.5 | 0.743 | 82.6 |             |
|        |     | m-Coumaric Acid, HCAD (67)| 0.633 | 0.814 | 24.2 | 0.239 | 27.0 | −0.02 | 20.3 |             |
|        |     | p-MPLA, HPLAD (85)       | 0.658 | 0.877 | 45.1 | −0.17 | 20.6 | 0.494 | 79.7 |             |
| C      | B   |                     |     |        |     |       |    |       |    |             |
|        |     | Isorhamnetin, Flavonol (12) | 0.536 | 0.991 | 100.0 | 0.946 | 79.7 | 0.986 | 94.7 | Kaempferol   |
|        |     | Kaempferol, Flavonol (14) | 0.547 | 0.991 | 100.0 | 0.994 | 100.0 | 0.997 | 100.0 |             |
|        |     | Hesperetin, Flavanone (18) | 0.667 | 0.251 | 5.5  | 0.076 | 31.0 | 0.275 | 45.6 |
|        |     | Naringenin, Flavanone (20) | 0.680 | 0.229 | 6.6  | 0.281 | 27.8 | 0.203 | 35.9 |
|        |     | m-Coumaric Acid, HCAD (67)| 0.633 | 0.814 | 24.2 | 0.239 | 27.0 | −0.02 | 20.3 |
|        |     | p-MPLA, HPLAD (85)       | 0.658 | 0.877 | 45.1 | −0.17 | 20.6 | 0.494 | 79.7 |
|        |     | p-Methoxy Phenyllactic Acid, HPLAD (85) | 0.536 | 0.897 | 40.0 | 0.021 | 9.8  | 0.821 | 70.8 |

Table 11. Results of Compound Matching for Test Compound C Using DB 1A.

| Name and Code | Rf 1 | H⁺ DEV 254 nm | H⁺ DEV 366 nm | H⁺ NP 366 nm | H⁺ T VSA | FL DEV λ | FI DEV λ | UV DEV λ₁ | UV DEV λ₂ | UV DEV λ₃ | FI VS λ | UV VS λ | Number of Potential Matches |
|---------------|------|---------------|---------------|--------------|----------|-----------|----------|------------|------------|------------|----------|----------|----------------------------|
| Test Compound C | 0.664 | 143           | 211           | 218          | 220      | 239      | 278      | 0          | 242        | 325        | 0        | 0        | 57                         |
| Hesperetin, (18) | 0.667 | 137           | 169           | 160          | 222      | 238      | 290      | 0          | 249        | 339        | 0        | 0        | 57                         |
| Naringenin, (20) | 0.680 | 137           | 177           | 160          | 223      | 246      | 292      | 0          | 249        | 333        | 0        | 0        | 57                         |
| Benzoic Acid, (40) | 0.663 | 136           | 185           | 159          | 211      | 241      | 276      | 0          | 246        | 277        | 0        | 0        | 57                         |
| Eudesmic Acid, (43) | 0.617 | 139           | 180           | 203          | 228      | 254      | 264      | 0          | 232        | 291        | 0        | 0        | 57                         |
| m-Toluic Acid, (51) | 0.681 | 137           | 160           | 189          | 221      | 242      | 284      | 0          | 243        | 287        | 0        | 0        | 57                         |
| o-Toluic Acid, (53) | 0.692 | 138           | 175           | 215          | 221      | 240      | 281      | 0          | 255        | 293        | 0        | 0        | 57                         |
| m-Coumaric Acid, (67) | 0.633 | 143           | 215           | 220          | 224      | 252      | 280      | 0          | 228        | 288        | 0        | 0        | 57                         |
| HVA, (79)       | 0.654 | 137           | 177           | 213          | 222      | 240      | 282      | 0          | 244        | 288        | 0        | 0        | 57                         |
| p-HPAA, (82)   | 0.620 | 138           | 179           | 180          | 222      | 239      | 277      | 0          | 249        | 285        | 0        | 0        | 57                         |
| p-MPLA, (85)   | 0.658 | 138           | 179           | 192          | 222      | 236      | 276      | 0          | 250        | 284        | 0        | 0        | 57                         |
| Name and Code | Rf 1 | H° DEV 254 nm | H° DEV 366 nm | Fl DEV λ | Fl DEV λm | UV DEV λ1 | UV DEV λ2 | UV DEV λ3 | Fl NP λ | UV NP λ1 | UV NP λ2 | UV NP λ3 |
|--------------|------|---------------|---------------|-----------|-----------|-----------|-----------|-----------|---------|-----------|-----------|-----------|
| Phloretic Acid, (87) | 0.630 | 141 | 188 | 190 | 221 | 232 | 278 | 0 | 0 | 254 | 288 | 0 | 0 |
| 2-M-4-VPh, (88) | 0.688 | 122 | 189 | 191 | 224 | 251 | 266 | 0 | 0 |  |
| p-Methoxyphenol, (89) | 0.685 | 134 | 171 | 235 | 223 | 231 | 275 | 254 | 263 |  |
| Vanillin, (99) | 0.637 | 136 | 222 | 224 | 219 | 238 | 278 | 0 | 0 |  |
| Pinobanksin, (24) | 0.697 | 137 | 191 | 174 | 224 | 239 |  |
| Formononetin, (32) | 0.637 | 147 | 195 | 194 | 223 | 250 |  |
| Cumic Acid, (41) | 0.697 | 136 | 180 | 185 | 221 | 246 |  |
| Gentisic Acid, (45) | 0.626 | 143 | 224 | 246 | 224 | 246 |  |
| Methyl Paraben, (47) | 0.676 | 128 | 179 | 220 | 224 | 252 |  |
| m-HBA, (50) | 0.644 | 138 | 181 | 184 | 223 | 245 |  |
| p-HBA, (54) | 0.637 | 130 | 184 | 180 | 225 | 252 |  |
| Resorcylic Acid, (56) | 0.630 | 144 | 214 | 226 | 224 | 249 |  |
| Salicylic Acid, (57) | 0.679 | 140 | 211 | 181 | 223 | 242 |  |
| CAPE, (63) | 0.676 | 140 | 235 | 209 | 225 | 250 |  |
| Ferulic Acid, (65) | 0.616 | 139 | 209 | 206 | 224 | 246 |  |
| Methyl Ferulate, (68) | 0.667 | 138 | 236 | 228 | 224 | 250 |  |
| Sinapic Acid, (74) | 0.630 | 139 | 224 | 198 | 222 | 245 |  |
| trans-p-CAME, (76) | 0.674 | 136 | 193 | 202 | 222 | 237 |  |
| 2-MBd, (96) | 0.709 | 134 | 188 | 194 | 222 | 253 |  |
| 2-Haph, (100) | 0.704 | 133 | 152 | 180 | 226 | 254 |  |
| 2-MHAPh, (101) | 0.669 | 139 | 241 | 204 | 225 | 251 |  |
| Isorhamnetin, (12) | 0.651 | 136 | 180 | 167 | 224 |  |
| Vanillic Acid, (59) | 0.623 | 136 | 192 | 209 | 228 |  |
| o-Coumaric Acid, (70) | 0.646 | 146 | 205 | 204 | 224 |  |
| p-Coumaric Acid, (71) | 0.630 | 136 | 199 | 205 | 223 |  |
| t-Cinnamic Acid, (75) | 0.672 | 140 | 242 | 222 | 259 |  |
| 3-PPA, (86) | 0.683 | 139 | 178 | 230 | 230 |  |
| 4-MPCat, (90) | 0.674 | 137 | 180 | 161 | 223 |  |
| Sakuranetin, (23) | 0.699 | 137 | 160 |  |
| Acetin, (3) | 0.649 | 135 | 180 |  |
| Apigenin, (4) | 0.617 | 134 | 199 |  |
| Chrysin, (6) | 0.672 | 132 | 186 |  |
| Genistein, (33) | 0.665 | 128 | 181 |  |
| Methyl-3,4,5-TMBz, (49) | 0.670 | 141 | 186 |  |
| p-MCA, (72) | 0.650 | 137 | 191 |  |
| Rosmarinic Acid, (73) | 0.630 | 131 | 175 |  |
| Phenylacetic Acid, (81) | 0.670 | 136 | 175 |  |
| Pyrocatechol, (95) | 0.662 | 137 | 181 |  |
| p-Anisaldehyde, (97) | 0.660 | 120 | 203 |  |
| Genkwanin, (7) | 0.653 | 139 |  |
| Kaempferide, (13) | 0.690 | 129 |  |
| Kaempferol, (14) | 0.654 | 131 |  |
| Quercetin, (16) | 0.617 | 126 |  |
| Biochanin A, (30) | 0.692 | 133 |  |
| DL-p-HPLA, (84) | 0.616 | 139 |  |

Legend: Rf1—retention factor in MPA, H° DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H° DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H° NP 366 nm—hue equivalent at 366 nm after derivatisation w/ NP-PEG-derivatisation reagent, Fl DEV λ—fluorescence λ max prior to derivatisation, Fl DEV λm—fluorescence λ min prior to derivatisation, UV DEV λ1-3—UV-Vis λ max prior to derivatisation, Fl NP λ—fluorescence λ max after derivatisation with NP-PEG reagent, UV NP λ1-3—UV-Vis λ max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.
### Table 12. Results of compound matching for test compound C using DB 1B.

| Name and Code                  | Rf1 | H' DEV 254 nm | H' DEV 366 nm | H' VSA 366 nm | Fl DEV | Fl DEV A | UV DEV A | UV DEV A2 | UV DEV A3 | F VSA | UV VSA |
|--------------------------------|-----|---------------|---------------|---------------|--------|----------|----------|-----------|-----------|--------|--------|
| test Compound C               | 0.635 | 140 | 183 | 204 | 14 | 221 | 239 | 278 | 0 | 0 | 246 | 349 |
| **Number of Potential Matches** | **64** | **64** | **61** | **58** | **52** | **50** | **37** | **18** | **14** | **14** | **10** |
| Hesperetin, (18)              | 0.667 | 137 | 169 | 202 | 20 | 222 | 238 | 290 | 0 | 0 | 250 | 375 |
| Naringenin, (20)              | 0.68 | 137 | 177 | 211 | 24 | 223 | 246 | 292 | 0 | 0 | 250 | 365 |
| Taxifolin, (25)               | 0.591 | 135 | 183 | 206 | 24 | 224 | 240 | 292 | 0 | 0 | 250 | 363 |
| Hesperetin, (18)              | 0.669 | 137 | 169 | 202 | 20 | 222 | 238 | 290 | 0 | 0 | 250 | 375 |
| Naringenin, (20)              | 0.68 | 137 | 177 | 211 | 24 | 223 | 246 | 292 | 0 | 0 | 250 | 365 |
| Taxifolin, (25)               | 0.591 | 135 | 183 | 206 | 24 | 224 | 240 | 292 | 0 | 0 | 250 | 363 |
| Benzoic Acid, (40)            | 0.663 | 136 | 185 | 199 | 18 | 211 | 241 | 276 | 0 | 0 | 250 | 371 |
| m-Toluic Acid, (51)           | 0.681 | 137 | 160 | 206 | 18 | 221 | 242 | 284 | 0 | 0 | 249 | 349 |
| m-Coumaric Acid, (67)         | 0.633 | 143 | 215 | 208 | 22 | 224 | 252 | 280 | 0 | 0 | 252 | 344 |
| p-MPLA, (85)                  | 0.658 | 138 | 179 | 228 | 20 | 222 | 236 | 276 | 0 | 0 | 250 | 400 |
| Eudesmic Acid, (43)           | 0.617 | 139 | 180 | 219 | 41 | 228 | 254 | 264 | 0 | 0 | 248 |
| HVA, (79)                     | 0.654 | 137 | 177 | 230 | 67 | 222 | 240 | 282 | 0 | 0 | 248 |
| Phloretic Acid, (87)          | 0.630 | 141 | 188 | 229 | 18 | 221 | 232 | 278 | 0 | 0 | 249 |
| p-Methoxyphenol, (89)         | 0.685 | 134 | 176 | 227 | 20 | 223 | 231 | 288 | 0 | 0 | 249 |
| Kaempferol, (14)              | 0.654 | 131 | 180 | 189 | 8 | 223 | 254 | 269 |
| VAME, (60)                    | 0.658 | 135 | 179 | 207 | 21 | 225 | 254 | 263 |
| Procatd, (98)                 | 0.594 | 135 | 232 | 240 | 29 | 221 | 240 | 283 |
| Vanillin, (99)                | 0.637 | 136 | 222 | 212 | 42 | 221 | 238 | 284 |
| Myricetin, (15)               | 0.587 | 130 | 132 | 189 | 40 | 224 | 253 |
| Quercetin, (16)               | 0.617 | 126 | 127 | 192 | 41 | 224 | 254 |
| Daidzein, (31)                | 0.600 | 137 | 161 | 226 | 29 | 222 | 251 |
| Formononetin, (32)            | 0.637 | 147 | 195 | 129 | 38 | 223 | 250 |
| Genistein, (33)               | 0.663 | 128 | 181 | 211 | 34 | 224 | 253 |
| 3,5-DHBA, (39)                | 0.594 | 138 | 191 | 222 | 34 | 225 | 251 |
| Gentisic Acid, (45)           | 0.626 | 143 | 224 | 213 | 22 | 224 | 246 |
| m-HBA, (50)                   | 0.644 | 138 | 181 | 232 | 42 | 223 | 244 |
| o-Anisic Acid, (52)           | 0.589 | 138 | 206 | 225 | 43 | 221 | 243 |
| p-HBA, (54)                   | 0.637 | 130 | 184 | 212 | 28 | 225 | 252 |
| Resorcylic Acid, (56)         | 0.630 | 144 | 214 | 202 | 57 | 224 | 249 |
| Salicylic Acid, (57)          | 0.679 | 140 | 211 | 205 | 48 | 223 | 242 |
| Caffeic Acid, (61)            | 0.589 | 139 | 211 | 215 | 35 | 224 | 250 |
| CAPE, (63)                    | 0.676 | 140 | 235 | 240 | 27 | 225 | 250 |
| Ferulic Acid, (65)            | 0.616 | 139 | 209 | 215 | 41 | 224 | 246 |
| Rosmarinic Acid, (73)         | 0.630 | 131 | 175 | 226 | 43 | 224 | 248 |
| Sinapic Acid, (74)            | 0.630 | 139 | 224 | 224 | 45 | 222 | 245 |
| Trans-p-CAME, (76)            | 0.674 | 136 | 193 | 205 | 49 | 222 | 237 |
| 2'-MAPh, (101)                | 0.669 | 139 | 241 | 188 | 0 | 225 | 251 |
| Acacetin, (3)                 | 0.649 | 135 | 180 | 204 | 34 | 224 |
| Apigenin, (4)                 | 0.617 | 134 | 199 | 206 | 46 | 224 |
| Chrysin, (6)                  | 0.672 | 132 | 186 | 190 | 47 | 224 |
| Isoflavone, (12)              | 0.651 | 136 | 180 | 191 | 43 | 224 |
| 2,3,4-TTHBA, (36)             | 0.589 | 138 | 185 | 226 | 21 | 223 |
| Methyl-3,4,5-TMBz, (49)       | 0.67 | 141 | 186 | 202 | 48 | 230 |
| Vanillic Acid, (59)           | 0.623 | 136 | 192 | 218 | 32 | 228 |
| o-Coumaric Acid, (70)         | 0.646 | 146 | 205 | 223 | 41 | 224 |
| p-Coumaric Acid, (71)         | 0.630 | 136 | 199 | 207 | 41 | 223 |
| p-MCA, (72)                   | 0.65 | 137 | 191 | 245 | 10 | 222 |
| t-Cinnamic Acid, (75)         | 0.672 | 140 | 242 | 202 | 44 | 229 |
| Pyrogallol, (94)              | 0.602 | 137 | 186 | 224 | 21 | 224 |
| Pyrocatechol, (95)            | 0.662 | 137 | 181 | 219 | 35 | 226 |
### Table 12. Cont.

| Name and Code                        | Rf1   | H^* DE 254 nm | H^* DE 366 nm | H^* NS 366 nm | H^* T VS | FID EV | FID EV m | UV DEV m | UV DEV 1 | UV DEV 2 | UV DEV 3 | Fl EV | Fl VS | UV VS |
|--------------------------------------|-------|---------------|---------------|---------------|----------|--------|----------|----------|-----------|-----------|-----------|-------|-------|-------|
| Methyl Syringate, (48)               | 0.610 | 138           | 175           | 219           |          | 23     |          |          |           |           |           |       |       |       |
| Phenylacetic Acid, (81)              | 0.67  | 136           | 175           | 242           | 23       |        |          |          |           |           |           |       |       |       |
| Methyl Paraben, (47)                 | 0.676 | 128           | 179           | 228           |          |        |          |          |           |           |           |       |       |       |
| CADE, (62)                           | 0.609 | 142           | 239           | 212           |          |        |          |          |           |           |           |       |       |       |
| Isoferulic Acid, (66)                | 0.600 | 140           | 227           | 215           |          |        |          |          |           |           |           |       |       |       |
| Methyl Ferulate, (68)                | 0.667 | 138           | 236           | 214           |          |        |          |          |           |           |           |       |       |       |
| 3-PAA, (86)                          | 0.683 | 139           | 178           | 231           |          |        |          |          |           |           |           |       |       |       |
| 4-MPCat, (90)                        | 0.674 | 137           | 180           | 242           |          |        |          |          |           |           |           |       |       |       |
| p-HPAA, (82)                         | 0.620 | 138           | 179           | 242           |          |        |          |          |           |           |           |       |       |       |
| p-Anisaldehyde, (97)                 | 0.66  | 120           | 203           |               |          |        |          |          |           |           |           |       |       |       |
| Abscisic Acid, (103)                 | 0.598 | 127           | 171           |               |          |        |          |          |           |           |           |       |       |       |
| Genkwanin, (7)                       | 0.653 | 139           |               |               |          |        |          |          |           |           |           |       |       |       |
| 2,3,4-TMBA, (37)                     | 0.602 | 142           |               |               |          |        |          |          |           |           |           |       |       |       |
| DL-p-HPLA, (84)                      | 0.616 | 139           |               |               |          |        |          |          |           |           |           |       |       |       |

Legend: Rf1—retention factor in MPA, H^* DE 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H^* DE 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H^* NS 366 nm—hue and colour equivalent at 366 nm after derivatisation w/ VSA-derivatisation reagent, H^* T VS—hue and colour equivalent at transmittance in white light after derivatisation w/ VSA-derivatisation reagent; FI DE 254 nm—fluorescence λmax prior to derivatisation, FI DE 254 nm m—fluorescence λmin prior to derivatisation, UV DEV 1-3—UV-Vis λmax prior to derivatisation, Fl VS—fluorescence λmax after derivatisation with VSA reagent, UV-Vis λ—UV-Vis λmax after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.

### Table 13. Results of compound matching for test compound C using DB 2A.

| Name and Code            | Rf2   | H^* DE 254 nm | H^* DE 366 nm | H^* NS 366 nm | FI DE 254 nm | FI DE 254 nm m | UV DEV 1 | UV DEV 2 | UV DEV 3 | FI NP 1 | FI NP 2 | FI NP 3 | UV NP 1 | UV NP 2 | UV NP 3 |
|--------------------------|-------|---------------|---------------|---------------|--------------|----------------|----------|----------|----------|---------|---------|---------|---------|---------|---------|
| Test Compound C          | 0.505 | 140           | 180           | 193           | 220          | 239            | 278      | 0        | 0        | 242     | 325     | 0       | 0       | 0       | 0       |
| Number of Potential Matches | 30   | 30            | 29            | 27            | 26          | 21             | 8        | 6        | 6        | 6       | 6       | 6       | 6       | 6       | 6       |
| Eudesmic Acid, (43)      | 0.504 | 139           | 180           | 203           | 228         | 254            | 264      | 0        | 0        | 232     | 291     | 0       | 0       | 0       | 0       |
| m-Coumaric Acid, (67)    | 0.493 | 143           | 215           | 220           | 224         | 252            | 280      | 0        | 0        | 228     | 288     | 0       | 0       | 0       | 0       |
| p-HPAA, (82)             | 0.471 | 138           | 179           | 180           | 222         | 239            | 277      | 0        | 0        | 249     | 285     | 0       | 0       | 0       | 0       |
| DL-p-HPLA, (84)          | 0.466 | 139           | 120           | 171           | 223         | 238            | 278      | 0        | 0        | 243     | 285     | 0       | 0       | 0       | 0       |
| p-MPLA, (85)             | 0.536 | 138           | 179           | 192           | 222         | 236            | 276      | 0        | 0        | 250     | 284     | 0       | 0       | 0       | 0       |
| Phloretic Acid, (87)     | 0.483 | 141           | 188           | 190           | 221         | 232            | 278      | 0        | 0        | 254     | 288     | 0       | 0       | 0       | 0       |
| Kaempferol, (14)         | 0.547 | 131           | 150           | 150           | 223         | 234            | 269      |          |          |         |         |         |         |         |         |
| Vanillin, (99)           | 0.548 | 136           | 222           | 224           | 221         | 238            | 284      |          |          |         |         |         |         |         |         |
| Formononetin, (32)       | 0.517 | 147           | 195           | 194           | 223         | 250            |          |          |          |         |         |         |         |         |         |
| Genistein, (33)          | 0.549 | 128           | 181           | 136           | 224         | 253            |          |          |          |         |         |         |         |         |         |
| 2,3,4-TMBA, (37)         | 0.479 | 142           | 120           | 147           | 225         | 254            |          |          |          |         |         |         |         |         |         |
| Cuminic Acid, (41)       | 0.525 | 136           | 180           | 185           | 221         | 246            |          |          |          |         |         |         |         |         |         |
| Gentisic Acid, (45)      | 0.508 | 143           | 224           | 246           | 224         | 246            |          |          |          |         |         |         |         |         |         |
| m-HBA, (50)              | 0.499 | 138           | 181           | 184           | 223         | 245            |          |          |          |         |         |         |         |         |         |
| o-Anisic Acid, (52)      | 0.499 | 138           | 206           | 193           | 221         | 243            |          |          |          |         |         |         |         |         |         |
| p-HBA, (54)              | 0.513 | 130           | 184           | 180           | 225         | 252            |          |          |          |         |         |         |         |         |         |
| Resorcylic Acid, (56)    | 0.504 | 144           | 214           | 226           | 224         | 249            |          |          |          |         |         |         |         |         |         |
| CADE, (62)               | 0.501 | 142           | 239           | 223           | 223         | 247            |          |          |          |         |         |         |         |         |         |
Table 13. Cont.

| Name and Code               | RI 2 | H² DEV 254 nm | H² DEV 366 nm | H² NP 366 nm | Fl DEV λ | Fl DEV λ m | UV DEV λ | UV DEV λ 1 | UV DEV λ 2 | UV DEV λ 3 | Fl NP λ | UV NP λ 1 | UV NP λ 2 | UV NP λ 3 |
|----------------------------|------|---------------|---------------|--------------|-----------|------------|----------|-------------|-------------|-------------|---------|-----------|-----------|-----------|
| CAPE, (63)                 | 0.554| 140           | 235           | 209          | 225       | 250        |          |             |             |             |         |           |           |           |
| Ferulic Acid, (65)         | 0.508| 139           | 209           | 206          | 224       | 246        |          |             |             |             |         |           |           |           |
| Isoferulic Acid, (66)      | 0.459| 140           | 227           | 216          | 224       | 248        |          |             |             |             |         |           |           |           |
| Isorhamnetin, (12)         | 0.536| 136           | 180           | 167          | 224       |            |          |             |             |             |         |           |           |           |
| Vanillic Acid, (59)        | 0.504| 136           | 192           | 209          | 228       |            |          |             |             |             |         |           |           |           |
| o-Coumaric Acid, (70)      | 0.319| 146           | 205           | 204          | 224       |            |          |             |             |             |         |           |           |           |
| p-Coumaric Acid, (71)      | 0.51 | 136           | 199           | 205          | 223       |            |          |             |             |             |         |           |           |           |
| 4-MPCAt, (90)              | 0.546| 137           | 180           | 161          | 223       |            |          |             |             |             |         |           |           |           |
| Methyl Syringate, (48)     | 0.524| 138           | 175           | 203          |           |            |          |             |             |             |         |           |           |           |
| Apigenin, (4)              | 0.507| 134           | 199           |              |           |            |          |             |             |             |         |           |           |           |
| Pyrocatechol, (95)         | 0.554| 137           | 181           |              |           |            |          |             |             |             |         |           |           |           |
| Genkwanin, (7)             | 0.533| 139           |               |              |           |            |          |             |             |             |         |           |           |           |

Legend: RI²—retention factor in MPB, H² DEV 254 nm—hue equivalent at 254 nm prior to derivatisation, H² DEV 366 nm—hue equivalent at 366 nm prior to derivatisation, H² NP 366 nm—hue equivalent at 366 nm after derivatisation w/ NP-PEG-derivatisation reagent, Fl DEV λ—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ 1-3—UV-Vis λ max prior to derivatisation, Fl NP λ—fluorescence λ max after derivatisation with NP-PEG reagent, UV NP λ 1-3—UV-Vis λ max after derivatisation with NP-PEG reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 14. Results of compound matching for test compound C using DB 2B.

| Name and Code               | RI 2 | H² DEV 254 nm | H² DEV 366 nm | H² NP 366 nm | Fl DEV λ | Fl DEV λ m | UV DEV λ | UV DEV λ 1 | UV DEV λ 2 | UV DEV λ 3 | Fl NP λ | UV NP λ 1 | UV NP λ 2 | UV NP λ 3 |
|-----------------------------|------|---------------|---------------|--------------|-----------|------------|----------|-------------|-------------|-------------|---------|-----------|-----------|-----------|
| Test Compound C             | 0.505| 140           | 183           | 204          | 14        | 221       | 239     | 278         | 0           | 0           | 246     | 349       |           |           |
| Number of Potential Matches | 15   | 15            | 12            | 11           | 8         | 7         | 0       | 0           | 0           | 0           | 0       | 0         | 0         | 0         |
| m-Coumaric Acid, (67)       | 0.493| 143           | 215           | 208          | 49        | 224       | 252     | 280         | 0           | 0           | 252     | 344       |           |           |
| p-MPLA, (85)                | 0.536| 138           | 179           | 228          | 38        | 222       | 236     | 276         | 0           | 0           | 250     | 400       |           |           |
| Eudesmic Acid, (43)         | 0.504| 139           | 180           | 219          | 41        | 228       | 254     | 264         | 0           | 0           | 248     |           |           |           |
| Phloretic Acid, (87)        | 0.483| 141           | 188           | 229          | 33        | 221       | 232     | 278         | 0           | 0           | 249     |           |           |           |
| Kaempferol, (14)            | 0.547| 131           | 150           | 189          | 8         | 223       | 254     | 269         |             |             |         |           |           |           |
| Vanillin, (99)              | 0.548| 136           | 222           | 212          | 42        | 221       | 238     | 284         |             |             |         |           |           |           |
| Formononetin, (32)          | 0.517| 147           | 195           | 179          | 38        | 223       | 250     |             |             |             |         |           |           |           |
| Genistein, (33)             | 0.549| 128           | 181           | 211          | 34        | 224       | 253     |             |             |             |         |           |           |           |
| Cumnic Acid, (41)           | 0.525| 136           | 180           | 205          | 2         | 221       | 246     |             |             |             |         |           |           |           |
| Gentisic Acid, (45)         | 0.508| 143           | 224           | 213          | 49        | 224       | 246     |             |             |             |         |           |           |           |
| m-HBA, (50)                 | 0.499| 138           | 181           | 232          | 38        | 223       | 254     |             |             |             |         |           |           |           |
| o-Anisic Acid, (52)         | 0.499| 138           | 206           | 225          | 43        | 221       | 243     |             |             |             |         |           |           |           |
| p-HBA, (54)                 | 0.513| 130           | 184           | 212          | 28        | 225       | 252     |             |             |             |         |           |           |           |
| Resorcylic Acid, (56)       | 0.504| 144           | 214           | 202          | 57        | 224       | 249     |             |             |             |         |           |           |           |
| CAPE, (63)                  | 0.554| 140           | 235           | 240          | 27        | 225       | 250     |             |             |             |         |           |           |           |
| Ferulic Acid, (65)          | 0.508| 139           | 209           | 215          | 41        | 224       | 246     |             |             |             |         |           |           |           |
| Apigenin, (4)               | 0.507| 134           | 199           | 206          | 46        | 224       |         |             |             |             |         |           |           |           |
| Isorhamnetin, (12)          | 0.536| 136           | 180           | 191          | 43        | 224       |         |             |             |             |         |           |           |           |
| Vanillic Acid, (59)         | 0.504| 136           | 192           | 218          | 32        | 228       |         |             |             |             |         |           |           |           |
| o-Coumaric Acid, (70)       | 0.519| 146           | 205           | 223          | 41        | 224       |         |             |             |             |         |           |           |           |
Table 14. Cont.

| Name and Code                   | Rf 2 | H’ DEV 254 nm | H’ DEV 366 nm | H’ VSA 366 nm | H’ T VSA | Fl DEV λ | Fl DEV λ m | Fl DEV λ 3 | UV DEV λ 1 | UV DEV λ 2 | UV DEV λ 3 | Fl VS λ | UV VSA |
|--------------------------------|------|---------------|---------------|---------------|-----------|-----------|-------------|-------------|-------------|-------------|-------------|-----------|---------|
| p-Coumaric Acid, (71)          | 0.51 | 136           | 199           | 207           | 41        | 223       |
| Pyrocatechol, (95)             | 0.554| 137           | 181           | 219           | 36        | 226       |
| Dibenzyl Oxalate, (102)        | 0.531| 139           | 199           | 245           | 62        | 222       |
| Methyl Syringate, (48)         | 0.524| 138           | 175           | 219           | 43        |
| CADE, (62)                     | 0.501| 142           | 239           | 212           |
| Isofleric Acid, (66)           | 0.459| 140           | 227           | 215           |
| 4-MPCat, (90)                  | 0.546| 137           | 180           | 242           |
| p-HPA, (82)                    | 0.471| 138           | 179           |               |
| Genkwanin, (7)                 | 0.533| 139           |               |               |
| 2,3,4-TMBA, (37)               | 0.479| 142           |               |               |
| DL-p-HPLA, (84)                | 0.466| 139           |               |               |

Legend: Rf2—retention factor in MPB, H’ DEV 254 nm—hue and colour equivalent at 254 nm prior to derivatisation, H’ DEV 366 nm—hue and colour equivalent at 366 nm prior to derivatisation, H’ VS 366 nm—hue and colour equivalent at 366 nm after derivatisation w/ VSA-derivatisation reagent, H’ T VS—hue and colour equivalent at transmittance in white light after derivatisation w/ VSA-derivatisation reagent; Fl DEV λ max—fluorescence λ max prior to derivatisation, Fl DEV λ m—fluorescence λ min prior to derivatisation, UV DEV λ 1-3—UV-Vis λ max prior to derivatisation, Fl VS λ—fluorescence λ max after derivatisation with VSA reagent, UV-Vis λ—UV-Vis λ max after derivatisation with VSA reagent. Note: coloured cells represent colours as seen on HPTLC plate.

Table 15. Consolidation of match compounds for test compound C.

| Unknown | Rf  | DB  | Name                              | Rf   | Match                              | Rf   |
|---------|-----|-----|-----------------------------------|------|------------------------------------|------|
|         |     | 1A  | hesperetin, (18)                  | 0.667| hesperetin, (18)                   | 0.667|
|         |     |     | naringenin, (20)                  | 0.680| naringenin, (20)                   | 0.680|
|         |     |     | benzoic acid, (40)                | 0.663| benzoic acid, (40)                 | 0.663|
|         |     |     | eudesmic acid, (43)               | 0.617| m-toluic acid, (51)                | 0.681|
|         |     |     | m-toluic acid, (51)               | 0.617| m-coumaric acid, (67)              | 0.633|
|         | 0.664| 1B  | o-toluic acid, (53)               | 0.692| p-MPLA, (85)                       | 0.658|
|         |     |     | m-coumaric acid, (67)             | 0.633|                                   |      |
|         |     |     | HVA, (79)                         | 0.654|                                   |      |
|         |     |     | p-HPA, (82)                       | 0.620|                                   |      |
|         |     |     | p-MPLA, (85)                      | 0.658|                                   |      |
|         |     |     | hesperetin, (18)                  | 0.667|                                   |      |
|         |     |     | naringenin, (20)                  | 0.680|                                   |      |
|         |     |     | benzoic acid, (40)                | 0.663|                                   |      |
|         |     |     | eudesmic acid, (43)               | 0.617|                                   |      |
|         |     |     | m-toluic acid, (51)               | 0.617|                                   |      |
|         |     |     | o-toluic acid, (53)               | 0.692|                                   |      |
|         |     |     | m-coumaric acid, (67)             | 0.633|                                   |      |
|         |     |     | p-MPLA, (85)                      | 0.658|                                   |      |
|         | 0.505| 2A  | m-coumaric acid, (67)             | 0.493|                                   |      |
|         |     |     | eudesmic acid, (43)               | 0.504|                                   |      |
|         |     |     | m-coumaric acid, (67)             | 0.493|                                   |      |
|         |     |     | p-HPA, (82)                       | 0.471|                                   |      |
|         |     |     | p-MPLA, (85)                      | 0.536|                                   |      |
|         |     |     | phloretic acid, (87)              | 0.483|                                   |      |
|         |     |     | m-coumaric acid, (67)             | 0.493|                                   |      |
|         |     |     | p-MPLA, (85)                      | 0.536|                                   |      |
|         | 0.536| 2B  | m-coumaric acid, (67)             | 0.493|                                   |      |

The same filtering methodology was then applied for the data generated for test compound A against database 1B. This reduced the initial set of potential matches from 33 to only 2 potential candidates, namely, methyl syringate and syringic acid (Table 3).

In the following step, spectral overlays were performed with the UV-Vis spectrum of test compound A and that of methyl syringate and syringic acid, respectively (Figure 1A).
Test-compound A featured an absorbance peak between 250 and 337 nm, and when overlaying and comparing this region with methyl syringate, a correlation of 0.993 was observed. For the syringic acid, the correlation was 0.994; thus, by applying the difference threshold of ±0.100, no discrimination between the quality of the match could be achieved. Similarly, the spectral overlay based on a ±0.125 AU range of the spectra of each match resulted in 95.5% of the absorbance values of the test compound in the investigated region to fall within that of the methyl syringate (Figure 1B), while the match was 100.0% for the syringic acid (Figure 1C). These findings were not outside the ±10% difference threshold set for matching, indicating that the UV-Vis spectral matching prior to derivatisation was insufficient for identifying the identity of the test compound.

The same spectral overlay approach was applied to the UV-Vis spectra obtained after derivatisation with NP-PEG reagent. Figure 2A shows the UV-Vis spectral overlay versus the consolidated matches. Test compound A was found to have a UV-Vis peak between 250 and 344 nm; the correlation in this region with methyl syringate was found to be 0.939,
while for syringic acid it was 0.986, again an insufficient difference to discriminate between the two standards based on a ±0.100 threshold difference. On visual inspection, a distinct shoulder in the spectrum of test compound A could be observed, a feature that is also present in the spectrum of syringic acid (Figure 2C), but not in that of methyl syringate (Figure 2B). When quantitatively analysed, only 78.9% of the absorbance values of test compound A fell within the ±0.125 AU of the signals of methyl syringate (Figure 2B), whereas 100.0% of its absorbance values were found to be within the ±0.125 AU of the signals of syringic acid (Figure 2C). Applying the ±10% difference threshold, it could thus be concluded that test compound A was most likely syringic acid, indicating that UV-Vis analysis after derivatisation with NP-PEG reagent was able to discriminate between the two remaining consolidated candidates.

Figure 2. (A–C): UV-Vis (after derivatised in NP-PEG reagent) spectra overlay of test compound A (UNK A) vs. methyl syringate and syringic acid and UV-Vis (after derivatised in NP-PEG reagent) spectra overlay of test compound A (UNK A) vs. ±0.125 AU of methyl syringate (B) and vs. ±0.125 AU of syringic acid (C).

Figure 3A shows the UV-Vis spectral overlay of test compound A after derivatisation with VSA reagent against the two consolidated candidate matches with methyl syringate yielding a matching correlation of 0.669, whereas that of syringic acid was 0.870. By applying
the difference threshold of ±0.100, it was found that test compound A had a spectral signature that was more similar to that of syringic acid. Additionally, by analysing the spectral overlays visually (Figure 3B,C), the wave inflections of test compound A were more like syringic acid when compared to methyl syringate. Quantitatively, only 60.5% of the absorbance values of test compound A fell within the ±0.125 AU of the signals of methyl syringate (Figure 3B), whereas the match was found to be 84.3% for syringic acid (Figure 3C). By applying the ±10% difference threshold, it could thus be concluded that test compound A was more likely to be syringic acid, indicating that the UV-Vis analysis after derivatisation with VSA reagent was also able to discriminate between the two consolidated potential matches.

Figure 3. (A–C): UV-Vis (after derivatised in VSA reagent) spectra overlay of test compound A (UNK A) vs. methyl syringate and syringic acid and UV-Vis (after derivatised in VSA reagent) spectra overlay of test compound A (UNK A) vs. the ±0.125 AU of methyl syringate (B) and vs. ±0.125 AU of syringic acid (C).

The fluorescence spectra of test compound A prior to and after derivatisation were also compared with the two potential matches, but they did not allow us to discriminate between them and therefore did not add any further information to the analysis.

The same filtering procedure as that described above in detail was followed for test compound A using data generated in MPB. Tables 4 and 5 illustrate how a list of potential...
matches was derived using derivatisation with NP-PEG (database 2A) or VS (database 2B), respectively. As can be seen, the database matching yields syringic acid as the sole match for test compound A, confirming the previous results. Post-analysis, the correct identification of test compound A was confirmed, validating the filtering process established for the database application.

2.2.2. Test Compound B

The same filtering approach as that described in Section 2.2.1 was conducted in order to ascertain the identity of test compound B. Using the data generated with MPA (Tables 6 and 7), isorhamnetin and kaempferol were identified as potential hits and were therefore considered as the consolidated matches for test compound B.

Corresponding data, generated using MPB, are shown in Tables 8 and 9. Isorhamnetin and kaempferol were once more identified as potential matches in this screening process.

In order to identify the correct match, various spectral overlays were performed (Figures S16–S18). The Pearson’s correlations as well as percent matches for each pair of databases are shown in Table 10.

Based on the derived correlations and percentage spectral matches, kaempferol was considered as a match for test compound B. A post-analysis of the correct identification of test compound B was confirmed, again validating the filtering process established for the database application.

2.2.3. Test Compound C

Test compound C served as a negative control to ensure that the database filtering approach and spectral overlay system did not yield false-positive hits. The data obtained following the various filtering steps are presented in Tables 11 and 12 using MPA and Tables 13 and 14 using MPB.

Table 15 illustrates that hesperetin, naringenin, benzoic acid, m-toluic acid, m-coumaric acid and p-methoxyphenyllactic acid are all identified as potential hits for test compound C and are therefore considered as consolidated matches, and based on the spectral overlays (Figures S19–S20), none of the standards in the database could be identified as matches for test compound C as only low correlations and percent spectral matches could be found (Table 10).

Post-analysis, test compound C was confirmed to be acetyl salicylic acid or aspirin (CAS No. 50-78-2, Chem Supply Australia Pty Ltd. (Port Adelaide, SA, Australia), a standard that was not included in the database, confirming the ability of the screening process to successfully avoid false-positive identifications.

2.2.4. HPTLC Analysis of Manuka Honey Extract

The organic extract of Manuka honey (*Leptospermum scoparium*) was analysed using HPTLC, and images were generated in the same way as for the standards and the artificial honey spiked with the three test compounds (Figures 4a and 5a). Images taken prior to derivatisation (A and B) were converted into corresponding chromatograms (Figures 4b and 5b) to determine major peaks and served as Rf references for the spectral scans that were performed.
derivatisation (A and B) were converted into corresponding chromatograms (Figure 4b and 5b) to determine major peaks and served as Rf references for the spectral scans that were performed.

Figure 4. HPTLC plate images (a) obtained under the following light conditions: 254 nm (prior to derivatisation A), 366 nm (prior to derivatisation B), 366 nm (after derivatised with NP-PEG-C), 366 nm (after derivatisation with VSA), transmittance in white light (after derivatisation with VSA-E) and chromatograms (b) of Manuka honey (L. scoparium) using mobile-phase A.

The Rf values, colour hues and λ max and λ min for the UV-Vis and fluorescence spectra obtained prior to and after derivatisation with the NP-PEG and VS reagents were tabulated for all the identified major bands in Manuka honey (Tables S15 and S16 for MPA and Tables S17 and S18 for MPB). The validated filtering process as outlined before was employed to determine the potential matches for these bands.

After identifying the consolidated matches, spectral overlays were performed in a similar way as described in previous sections. A summary of the correlations and percent matches is presented in Table 16.

In summary, by using databases 1A and B, the band at Rf 0.02 was identified as leptosperine (46, see Table S8 and Figure S8 for structure), the band at Rf 0.199 as mandelic acid (80, see Table S10 and Figure S11 for structure) and the band at Rf 0.240 as kojic acid (105, see Figure S15 for structure). The band at Rf 0.319 was identified as lepteridine (106, see Figure S15 for structure), Rf 0.392 as epigallocatechin gallate (EGCG), (29, see Table S5 and Figure S5 for structure), Rf 0.460 as lumichrome (107, see Figure S15 for structure) and the band at Rf 0.623 as methyl syringate (48, see Table S8 and Figure S8 for structure).
Figure 5. HPTLC plate images (a) obtained under the following light conditions: 254 nm (prior to derivatisation A), 366 nm (prior to derivatisation B), 366 nm (after being derivatised with NP-PEG-C), 366 nm (after being derivatised with VSA-D), transmittance in white light (after being derivatised with VSA-E) and chromatograms (b) of Manuka honey (L. scoparium) using mobile-phase B.

By using databases 2A and 2B, the band at Rf 0.150 was identified as kojic acid, the band at Rf 0.220 as lepteridine, the band at Rf 0.310 as gallic acid (44, see Table S8 and Figure S8 for structure) and the band at Rf 0.349 as mandelic acid. The band at Rf 0.425 was identified as 2,3,4-trihydroxybenzoic acid (36, see Table S8 and Figure S8 for structure), the band at 0.470 as o-anisic acid (52, see Table S8 and Figure S8 for structure), the band at Rf 0.513 as methyl syringate and, finally, the band at Rf 0.603 as salicylic acid.

Ideally, the matches identified in one database set (DBs 1A and 1B) should also be identified in the second database set (DBs 2A and 2B). This ‘double identification’ was possible for kojic acid, lepteridine, methyl syringate and lumichrome. However, given the complexity of honey as a natural product and the presence of a multitude of compounds in the investigated honey extract, well separated and thus potentially identifiable bands in one solvent system might overlap with bands in another solvent system, with a poor band resolution making compound identification impossible. In this light, it is plausible that some matches were only found in one but not the second database set. In the case of Manuka honey, this was the case for leptosperine, mandelic acid, epigallocatechin gallate, lumichrome, 2,3,4-trihydroxybenzoic acid, o-anisic acid and salicylic acid. The richness
of the data generated by HPTLC analysis and explored in the methodological compound-identification approach outlined in this study thus offered various avenues to successfully match compounds against the established databases.

Table 16. Summary of the correlations and % spectra matches used to determine the identity of the unknown bands for Manuka honey.

| Database          | UNK | RF  | Name, Class and Code                  | RF  | UV DEV | %  | UV NP | %  | UV VS | %  | Match         |
|-------------------|-----|-----|---------------------------------------|-----|--------|----|-------|----|-------|----|---------------|
|                   | 1   | 0.020 | leptosperine, HCAD (46)               | 0.014 | 0.863  | 22.4 | −0.013 | 49.5 | 0.923 | 78.3 | leptosperine  |
|                   | 2   | 0.077 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 3   | 0.134 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 4   | 0.199 | mandelic acid, HPAAD (80)             | 0.162 | 0.696  | 65.9 | 0.882  | 43.4 | 0.911 | 75.1 | mandelic acid|
|                   | 5   | 0.240 | kojic acid, non-phenolic (105)        | 0.287 | 0.960  | 26.4 | 0.518  | 31.1 | 0.847 | 26.0 | kojic acid    |
|                   | 6   | 0.319 | lepteridine, non-phenolic (106)       | 0.314 | 0.924  | 44.3 | 0.902  | 50.4 | 0.526 | 47.0 | lepteridine   |
|                   | 7   | 0.392 | EGCG, flavan-3-ol (29)                | 0.407 | 0.834  | 82.0 | 0.479  | 39.4 | 0.837 | 55.5 | EGCG          |
|                   | 8   | 0.467 | lumichrome, non-phenolic (107)        | 0.464 | 0.319  | 25.1 | 0.481  | 62.9 | 0.231 | 27.4 | lumichrome    |
|                   | 9   | 0.513 | -                                     | -    | -      | -   | -      | -   | -      | -   | None          |
|                   | 10  | 0.543 | -                                     | -    | -      | -   | -      | -   | -      | -   | None          |
|                   | 11  | 0.635 | methyl syringate, HBAD (48)           | 0.610 | 0.983  | 48.4 | 0.944  | 70.5 | 0.907 | 77.6 | methyl syringate|
|                   | 12  | 0.685 | benzoic acid, HBAD (40)               | 0.663 | 0.894  | 30.3 | 0.043  | 15.9 | 0.413 | 17.1 | none          |
| 2A and 2B         | 1   | 0.021 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 2   | 0.081 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 3   | 0.098 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 4   | 0.121 | -                                     | -    | -      | -   | -      | -   | -      | -   | none          |
|                   | 5   | 0.150 | kojic acid, non-phenolic (105)        | 0.171 | 0.939  | 30.2 | 0.914  | 61.1 | 0.674 | 21.4 | kojic acid    |
|                   | 6   | 0.220 | lepteridine, non-phenolic (106)       | 0.217 | 0.514  | 25.4 | 0.947  | 33.6 | 0.674 | 43.1 | lepteridine   |
|                   | 7   | 0.310 | gallic acid, HBAD (44)                | 0.321 | 0.969  | 84.1 | 0.894  | 73.3 | 0.912 | 87.9 | gallic acid   |
|                   | 8   | 0.349 | mandelic acid, HPAAD (80)             | 0.347 | 0.438  | 36.4 | 0.027  | 22.9 | 0.892 | 68.3 | mandelic acid|
|                   | 9   | 0.425 | 2,3,4-THBA, HBAD (36)                 | 0.437 | 0.828  | 31.8 | 0.963  | 86.8 | 0.753 | 51.2 | 2,3,4-THBA    |
|                   | 10  | 0.470 | m-HBA, HBAD (50)                      | 0.499 | 0.904  | 37.4 | 0.961  | 27.5 | −0.518 | 20.3 | o-anisic acid |
|                   | 11  | 0.513 | methyl syringate, HBAD (48)           | 0.524 | 0.992  | 56.0 | 0.979  | 100.0 | 0.930 | 85.1 | methyl syringate|
|                   | 12  | 0.603 | pinobanksin, flavanonol (24)          | 0.591 | 0.760  | 24.0 | 0.655  | 35.9 | 0.782 | 35.9 | salicylic acid|
|                   |     |       | salicylic acid, HBAD (57)             | 0.582 | 0.771  | 15.9 | 0.909  | 13.1 | 0.545 | 75.1 |               |
Leptideridine has been previously reported in Manuka honey and is considered one of the honey’s biomarkers [26,27,35,42]. Gallic acid was also found to be present in Manuka honey, which was previously reported [21,24,26,31,33,43], along with methyl syringate [25,26,28,29,31,33,35,44], lumichrome [26,29,45], leptosperine [25–27,35], salicylic acid [29,46], o-anisic acid [19,26,27,29,34,44], 2,3,4-trihydroxybenzoic acid [21] and kojic acid [44]. This study was, however, the first to report the presence of mandelic acid and epigallocatechin in Manuka honey.

2.2.5. Validation by HPLC

HPLC with a photodiode array detector (DAD or PDA) [20,21,25–27,29,34,42,47,48], and UV or UV/UV detector [19,22,24,28,49] were found to be the most commonly used instrumentations in the identification of phenolic compounds in honey, followed by LC-MS [31,33,46]. A combination of HPLC, LC-MS and GC-MS [44], and also fluorescence spectroscopy, were used in the study [33]. Given the popularity of HPLC with photodiode array detection for determining phenolic constituents in honey, a cross-validation of the findings of this study with the data obtained with this instrumentation was performed.

The validation was conducted at the University of the Sunshine Coast Honey Research Laboratory using HPLC DAD (protocol not published). Based on this analysis, Leptosperin was detected as the most abundant compound (522 ppm), followed by 3-phenyllactic acid or DL-β-phenyllactic acid (226 ppm), methyl syringate (60 ppm), 2-methoxybenzoic acid or o-anisic acid (54 ppm), kojic acid (34 ppm), pyrogallol (13 ppm), lepteridine (8 ppm), p-hydroxyphenyllactic acid (5 ppm), syringic acid (3 ppm) and lumichrome (3 ppm).

Of these identified Manuka honey constituents, 3-phenyllactic acid or DL-β-phenyllactic acid, pyrogallol, p-hydroxyphenyllactic acid and syringic acid were not detected by HPTLC, although these standards were part of the database used for filtering. On the other hand, salicylic acid, 2,3,4-THBA, mandelic acid and EGCG were not detected by HPLC-DAD, but could be identified in the HPTLC analysis. These differences might be attributed to the fact that for the HPLC analysis, the honey sample in its entirety was analysed as an aqueous solution, whereas in the case of the HPTLC analysis, an organic extract of the honey was utilised. The solvent used in the extraction of the non-sugar constituents in honey was dichloromethane: acetonitrile (1:1) that might not have extracted all non-sugar constituents present. In a study conducted by Stanek and Jasicka-Misiak [20], acidified Manuka honey (pH 2 with HCl) was extracted using Amberlite XAD-2 in order to obtain its phenolic constituents. From this extract, rosmarinic acid, ellagic acid, p-coumaric acid and myricetin were qualitatively determined based on colour and Rf values, indicating that the extraction method can play a significant role in the type of compounds that are detected in the analysis.

3. Discussion

The proposed HPTLC-based methodology offered advantages over other compound identification strategies. Two detection conditions, namely, excitation at 254 nm and at 366 nm, are commonly used in HPTLC prior to compound derivatisation. Phenolics can be detected by quenching the adsorbent material’s fluorescence (254 nm) and/or after UV irradiation (366 nm), with the latter being considered the most sensitive detection method in HPTLC. Detection modes are in the form of images from which rich data can be derived, particularly the colour and Rf values of the bands.

The colours of bands in HPTLC analysis have been used in the qualitative and quantitative analyses of honey; however, they were only described using basic colour descriptions [20,50,51]. This study was able to capture more nuanced colour differences in the bands by converting their RGB values into a single hue (H°) value. This conversion allowed the RGB values to be easily captured, expressed by a single value that facilitated inter-band colour comparisons. It was also observed that certain groups of compounds or compounds that contained the same functional groups tended to present distinct, bright-colour or fluoresce patterns that might be used to predict their compound class. For example, flavones,
such as acacetin (3), apigenin (4), chrysin (6), gekwanin (7) and vitexin (9), have one active site located at the 5-hydroxy-4-keto group, and they all had hue values ranging from 61.5° to 121.6° (yellow to green).

Since these imaging conditions without derivatisation might, nonetheless, not capture all compounds, those that are not detectable at 254 or 366 nm can be visualised after a reaction with a suitable reagent; in this study, either VSA or NP-PEG [52]. In HPTLC-based honey research, to date, 1% methanolic AlCl₃, ceric phosphomolybdate, 2-aminoethyl diphenylborate, vanillin sulfuric acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) appear to be the most popular spraying reagents [20,37,50–53]. In this study, 2-aminoethyl diphenylborate (natural product reagent or Neu’s reagent) and vanillin sulfuric acid were used to derivatise the honey constituents for visualisation. The derivatisation did not only enhance the visualisation of the samples, but also enhanced their UV-Vis and fluorescence spectra. Since compounds might react differently to different spraying reagents, using two derivatising reagents increased the chances of enhanced visualisation and ultimately compound matching. The rich data generated in the RGB and subsequent hue values of the various honey bands at 254 nm, and 366 nm as well as at 366 nm and white light after derivatisation with VS and NP-PEG reagent thus allowed for higher levels of certainty in the compound identification compared to the analytical approaches (e.g., HPLC-DAD) that were unable to tap into this rich resource of information for screening and filtering purposes to discriminate between structurally often very similar honey constituents.

The study also used two different mobile phases to ensure that a large number of extracted honey bands could be adequately accounted for. Using MPA, six compounds could be identified in Manuka honey; with the slightly less polar MPB, the chemical identities of eight compounds could be determined.

Furthermore, an additional advantage of HPTLC-derived Rf values as primary screening criterion compared to other identification techniques relying on chromatographic separation of compounds (e.g., HPLC) is that no Rf drift over time associated, for example, with an aging of column material can be encountered. Rf values in the database are therefore more stable and are able to be used for long-term screening studies, as in every experiment a fresh stationary phase will be used and therefore changes over time in retention factors will not occur.

In this study, the fluorescence λ max and λ min after development, UV-Vis λ max after development and after derivatisation, and the fluorescence λ max after derivatisation were all also been found to be important tools in narrowing the number of possible matches for an unknown sample. A small tolerance range (±15 nm) was employed as a filtering criterion, except for UV-Vis spectra after derivatisation (±60 nm), in order to take into consideration a possible change in pH and other parameters, which can lead to slight bathochromic or hypsochromic shifts. Although the use of HPTLC is not new in the analysis of phenolic compounds in honey [5,8,20,50,52,54,55], to our knowledge, this study was the first to report the use of a HPTLC scanner for honey-compound determination. It was found that UV-Vis spectra after development alone could not always univocally determine the identity of a compound; therefore, UV-Vis spectra after derivatisation were also utilised. These, especially after derivatisation with NP-PEG reagent, were found to be a very important discriminating tool in determining the unknown sample’s identity. Compared with structure determination methods that solely rely on UV-Vis identification (e.g., HPLC-DAD), HPTLC-based screening can thus offer a distinct advantage.

The “gold standard” for phenolic compound determination is ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS) [56]. However, it is a costly technique, both in terms of equipment and running costs, and it requires technical expertise for its operation and data analysis [57]. In light of these shortcomings, high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) appears to be the most commonly employed technique for the qualitative and quantitative analyses of phenolic compounds [1,8,16,58]. Although the set up of HPLC-DAD is relatively cheap and the analysis robust, the method nonetheless faces several
disadvantages, such as i) compound identification is only based on retention times and associated UV spectra, which might not allow us to sufficiently discriminate between compounds of a very similar chemical nature, and ii) low detection and quantification limits when analysing complex matrices [59].

The spectral overlay system that was developed, along with the use of correlation and percent-match calculations of the inflection of the unknown against potential compound matches, was also found to be a very useful tool not only in differentiating between multiple potential matches, but also in confirming the identity of the unknown ones. While studies employing DA detection might also be able to tap into spectral characteristics beyond simple λ max information, the availability of multiple spectra (UV-Vis and fluorescence spectra prior and after derivatisation) offers much richer data and therefore a better chance of univocally discriminating between potential matches of similar chemical characteristics that might not be able to be differentiated using a single UV-Vis spectrum. A case in point is unknown A where, based on the spectral overlay of the UV-Vis spectrum prior to derivatisation, a distinction between the two candidate matches of methyl syringate and syringic acid was not possible; however, on closer inspection of their UV-Vis spectra after derivatisation with NP-PEG reagent, it could be determined that syringic acid was the correct match.

While compound matching using the database and the developed filtering approach is a multi-step process, many of the individual steps can be automated, for example, the generation of hue values, spectral overlays (using pre-modelled Excel® worksheets) as well as the calculation of correlations and % agreement in absorbance values.

Despite the significant advantages over other compound identification methods, some limitations of the HPTLC-derived database system for the qualitative analysis of honey constituents need to be acknowledged. The approach relies on an extensive set of data, which requires a considerable amount of time to acquire, particularly the four scanning steps and the manual entry of RGB values.

It is also important to note that the derivatising process, particularly when using VSA reagent, is highly time dependent and must therefore be conducted in a controlled manner to generate reproducible results. The HPTLC development itself is also dependent on moisture levels, which can affect the Rf value of the bands. As the Rf value is used as primary screening criterion, the development of the plate should thus be performed in a humidity-control development chamber. To address this potential limitation, Rf ±0.05 was set as the primary filtering criterion in the database search to allow for slight run to run variations.

Another limitation of the use of HPTLC in the analysis of phenolic compounds in honey is that, by default, the photodocumentation is restricted to the use of only two wavelengths, namely, 254 nm and 366 nm. This means that prior to derivatisation, only those compounds that absorb at these wavelengths (254 nm for most simple phenolic compounds and 366 nm for most flavonoids) will be detected. Most caffeic acid derivatives, for example, present λ max values of around 320 nm; therefore, this compound class is difficult to identify using default detection. However, with the use of two derivatisation reagents, it was possible to overcome this limitation.

The development of the database used in this study can easily be adopted in another laboratories, as long as the HPTLC instrumentation and conditions indicated in the Methodology Section are properly replicated.

The database has at this point only been developed for qualitative analysis, and thus the limit of detection has not yet been determined; the system has, however, been validated for a reliable identification of phenolic compounds in honey and has been demonstrated to be a powerful tool preceding potential quantification experiments.

4. Materials and Methods

4.1. Chemicals and Reagents

In general, chemical standards were purchased from Ajax Finechem Pvt. Ltd. (Sydney, Australia), AK Scientific, Inc. (Union City, CA, USA), Alfa Aesar (Lancashire, UK),
Angene International Ltd. (Nanjing, China), Chem Supply Australia Pty Ltd. (Port Adelaide, SA, Australia), Combi-Blocks Inc. (San Diego, CA, USA), Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, Hubei, China), Sigma Aldrich (Castle Hill, NSW, Australia) and Sigma-Aldrich (St. Louis, MO, USA). Figures S1–S15 and Tables S1–S14 summarise information, such as the identity, supplier, prepared concentration and HPTLC sample application of the standard compounds used to construct the database. Compounds were selected based on the findings of a comprehensive review of (mainly) phenolic compounds identified, to date, in honeys around the world [16]. The pool of standards was complemented with isomers of some of these compounds, compounds that were reported for other bee products (e.g., pollen and propolis) and other phenolic compounds available to the research team. The standards were grouped in line with common phenolic compound classifications [16,60–62].

Other reagents used in the study were purchased from the following suppliers: Anhydrous magnesium sulphate (7487-88-9), ethanol (64-17-5), 2-aminoethyl diphenylborinate (524-95-8), glucose (D-glucose anhydrous, 50-99-7) and sucrose (57-50-1) from Chem Supply Australia Pty Ltd. (Port Adelaide, SA, Australia); vanillin (121-33-5) from Sigma-Aldrich (St. Louis, MO, USA); naringenin (67604-48-2) from Alfa Aesar (Lancashire, UK); ethyl acetate (141-78-6) and formic acid (64-18-6) from Ajax Finechem Pvt. Ltd. (Sydney, Australia); fructose (57-48-7) and maltose (6363-53-7) were purchased from Sigma Aldrich (Truganina, VIC, Australia); toluene from APS Chemicals (Sydney, Australia); dichloromethane (75-09-2), acetonitrile (75-05-8) and HPTLC Silica gel 60 F254 Plates 10 × 20 cm from Merck KGaA (Darmstadt, Germany); methanol (67-56-1) from Scharlau (Barcelona, Spain); PEG (25322-68-3) from PharmAust Manufacturing (Welshpool, Western Australia) and sulfuric acid (7664-93-9) from Merck KGaA (Darmstadt, Germany).

4.2. Honey Sample

Manuka honey (Leptospermum scoparium) was purchased from a local honey supplier in Queensland. No further authentication was performed to confirm its floral origin.

4.3. Preparation of Standards and Reagents

4.3.1. Standards

All standards used in the study were dissolved in methanol to concentrations as indicated in Tables S1–S14. Naringenin (0.5 mg/mL in methanol) was used as the HPTLC reference standard.

4.3.2. Developing Solvent

Two different mobile phases were used in the study: (a) toluene:ethyl acetate:formic acid (2:8:1, v/v/v), referred to as MPA, and (b) toluene:ethyl acetate:formic acid (6:5:1, v/v/v) referred to as MPB.

4.3.3. Derivatising Reagents

Natural product (NP)-derivatising reagent was prepared by dissolving 1 g of 2-aminoethyl diphenylborinate in methanol and then the volume of the solution was made up to 100 mL (1% m/v) [63]. Polyethylene glycol (PEG-400) reagent was prepared by mixing 5 g of polyethylene glycol in ethanol and then the volume of the solution was made up to 100 mL (5% m/v) [63]. Vanillin sulfuric acid (VSA)-derivatising reagent was prepared by adding 2 mL of sulfuric acid to 100 mL 1% vanillin solution (1 g/100 mL in ethanol) [36,52,64]. All derivatising reagents were stored at 0 °C when not in use.

4.3.4. Artificial Honey

An artificial honey solution was prepared by diluting 2 g of a sugar stock solution (21.625 g of fructose, 18.125 g of glucose, 1.000 g of maltose, 0.750 g of sucrose and 8.500 g of water) to 5 mL (40%) with deionised water. The solution was stored at 0 °C and used within a week [65].
4.4. Extraction of Non-Sugar Components in Honey

Each honey (1 g) was mixed with 2 mL of deionised water in stoppered glass test tubes and vortexed to facilitate dissolution and mixing. The resulting aqueous honey solutions were then extracted 3 times with 5 mL of a mixture of dichloromethane and acetonitrile (1:1, v/v). The respective organic extracts were combined and dried with anhydrous MgSO₄, filtered and then evaporated to dryness at 35 °C. The organic honey extracts were stored at 4 °C until analysis for which they were reconstituted with 100 µL methanol.

Artificial honey solutions containing 1% (m/v) of standards A and B, which were included in the database, were prepared as positive controls, and another artificial honey solution containing 1% (m/v) of standard C, which was not in the database, served as a negative control in the validation of the filtering approach used in the database application.

4.5. Chromatography and Derivatisation

Each standard and the respective honey/artificial honey extracts were chromatographed and derivatised as follows: duplicate plates were developed in mobile-phase MPA and were derivatised using either NP-PEG or VSA reagents (for details see Section 4.5.3). Duplicate plates were also run using mobile-phase MPB and again derivatised with NP-PEG and VSA, respectively. MPA was selected as a mobile phase because previous studies of honey using HPTLC analysis employed this mobile phase, allowing for cross-references to previous studies. MPB, with a slightly higher polarity, was chosen to ensure that more polar phenolics were also adequately separated and detected. In a similar way, VSA was employed as a derivatisation reagent as it had been used in numerous, previous HPTLC-based honey analyses [36,37,66], allowing for cross-referencing, whereas NP-PEG was selected as a versatile derivatisation reagent particularly suitable for the identification of phenolic compounds [67], which also allowed us to broadly differentiate between flavonoids and other phenolics based on colour development [39,67].

4.5.1. Sample Application

For the naringenin reference standard, 4 µL was applied, and for honey and artificial honey extracts, 7 µL. The application volumes for the various standards varied (Tables S1–S14). All samples were applied as 8 mm bands, 8 mm from the bottom of the HPTLC plate at a rate of 150 nL s⁻¹ using a semi-automated HPTLC sample applicator (Linomat 5, CAMAG).

4.5.2. Plate Development

The chromatographic separation was performed on HPTLC plates (20 cm × 10 cm glass-backed silica gel 60 F₂₅₄ plates) in an activated (MgCl₂·6 H₂O, 33–38% relative humidity) automated twin-trough (20 × 10 cm) development chamber (ADC2, CAMAG). The system was saturated for 15 min using saturation pads and the plates were first preconditioned with the mobile phase for 5 min, automatically developed to a distance of 70 mm at room temperature and then dried for 5 min. The developed plates were then photo-documented using the HPTLC imaging device (TLC Visualizer 2, CAMAG) under 254 nm, 366 nm and white light in transmittance mode (T). The entire chromatographic process as well as digital image processing and analyses were controlled by specialised HPTLC software (VisionCATS 3.1, CAMAG).

4.5.3. Derivatisation

To derivatise samples using NP-PEG reagent, the plates were first sprayed with 3 mL of 1% NP reagent (CAMAG Derivatiser, green nozzle at level 3) and were then allowed to dry for 5 min at 40 °C (CAMAG TLC Plate Heater III). The plates were then derivatised again using 5% PEG reagent (blue nozzle at level 2) followed by drying (CAMAG TLC Plate Heater III) for 5 min at 40 °C. The derivatised plates were analysed at 366 nm.

To derivatise plates using VSA reagent, they were sprayed with 3 mL of 1% vanillin sulphuric acid reagent (CAMAG Derivatiser, yellow nozzle at level 3), then heated (CA-
MAG TLC Plate Heater III) at 115 °C for 3 min. The derivatised plates were analysed at 366 nm and T white light after cooling down for 2 min.

4.5.4. Scanning of Individual Bands

Chromatograms of all standards and samples were generated in the various conditions and the major peaks were automatically determined by the software. A TLC Scanner 3 (CAMAG, Muttenz, Switzerland) was used to scan the spectra of each standard and the individual, to identify significant bands in both the UV-Vis (190–900 nm) and fluorescence excitation (190–380 nm) modes. The scans were performed using the following settings: dimension 5 × 0.2 mm (micro), optimisation set for maximum resolution, scanning speed set at 20 nm/s and K400 optical filter. Deuterium (190–380 nm) and tungsten (380–900 nm) were used as lamps and the fluorescence excitation mode was set at 380 </ 400 nm scans, and the emission was observed at 190–270 nm. Scanning was performed twice, prior and after derivatisation.

4.5.5. Systems Suitability Test (SST)

As a quality control step, a system suitability test (SST) was built into the analysis of all plates used both in the database development and also the qualitative determination of phenolic compounds. Only the results from plates that passed the set threshold of ±0.05 for the Rf and the minimum height for MPA (Rf 0.690, minimum height 0.108) and MPB (Rf 0.550, minimum height 0.120) were used.

4.6. Data Tabulation

Rf values in the two mobile phases used colours (expressed as RGB values) in the various image conditions (254 nm, 366 nm and T white light prior to derivatisation and 366 nm and T white light after derivatisation with NP-PEG and VSA reagent), UV-Vis and fluorescence spectra prior to and after derivatisation were recorded for all standards as well as the honey/artificial honey extracts.

In addition, colours expressed as RGB values were converted into hue values using the following formula:

$$\text{HUE (°)} = \text{IF}(180/\pi) \ast \text{ATAN2}(2 \ast A3 - B3 - C3, \sqrt{3} \ast (B3 - C3)) < 0, 180/\pi \ast \text{ATAN2}(2 \ast A3 - B3 - C3, \sqrt{3} \ast (B3 - C3)) + 360, 180/\pi \ast \text{ATAN2}(2 \ast A3 - B3 - C3, \sqrt{3} \ast (B3 - C3)))$$

where A = Red value, B = Green value and C = Blue value (https://www.mrexcel.com/board/threads/rgb-to-hue-formula.559852/, accessed on 30 September 2022).

Hue values were included in the database in lieu of RGB values as it allowed us to express colour by a single number. These hue values were further aligned with the colour family based on the following conversions: 0.00–29.99 = Red (R), 30.00–59.99 = Orange (O), 60.00–89.99 = Yellow (Y), 90.00–119.99 = Yellow Green (YG), 120.00–149.99 = Green (G), 150.00–179.99 = Turquoise (T), 180.00–209.99 = Cyan Blue (CB), 210.00–239.99 = Blue (B), 240.00–269.99 = Violet (V), 270.00–299.99 = Purple (P), 300.00–329.99 = Magenta (M), 330.00–359.99 = Scarlet (S) (https://www.blog.jimdoty.com/?p=11507, accessed on 30 September 2022).

4.7. Database Establishment

Four sub-databases were established (DB 1A and 1B, and 2A and 2B), one for each solvent system as well as the respective derivatisation agent used in the analysis.

4.8. Database Search Strategies

To match the unknown honey bands with potential standards included in the database for compound identification, a comprehensive strategy was set in place (Figure 6). A sort and filter feature was formulated so that the database automatically returned potential match compounds based on the information provided for the unknown.
**Figure 6.** Database filtration and spectra overlay protocol for determining the identity of an unknown sample using the developed HPTLC-based database.

**Rf value.** Rf values of unknown bands ± 0.05 were used as primary search criterion to generate a first list of potential hits from the database.

**Hue values.** Hue values were then used to narrow down further the list of potential hits using the hue value of the unknown band ± 60 as additional filter criterion.

**Fluorescence λ max and λ min, and UV-Vis λ max.** In a next step, we tried to match the fluorescence λ max and λ min, and the UV-Vis λ max of unknown honey bands prior to derivatisation, and the number of λ max/peaks in the UV-Vis spectra prior to derivatisation with that of any of the remaining potential hits in the database using λ ± 15 nm as the filtering criterion. This was followed by a potential matching with fluorescence λ max after derivatisation with λ ± 15 nm as the filtering criterion, and finally, with the UV-Vis λ max after derivatisation with λ ± 60 nm as filtering criterion.

To perform spectral matching with the identified potential hits, an automated spectral overlay system (see Section 4.9) was developed using Excel®, where spectral regions around the respective λ values of the unknown honey band (+/− 15 nm) were examined and the spectra considered to be a close match with a standard if the unknown band’s normalised absorbance fell within +/− 0.125 of the normalised absorbance of the standard identified as a potential hit.
If at this stage a final identification could not be performed because more than one standard in the database met the above search criteria, the respective fluorescence spectra prior to as well as after derivatisation with VS and NP-PEG were also investigated for potential matches adopting the screening process as described above.

4.9. Spectral Overlay System using Excel

Although the maxima of fluorescence and UV-Vis absorbance prior to and after derivatisation, and the minima of fluorescence prior to derivatisation were used to determine the identity of the unknown bands, these were found to be insufficient to confirm the identity of compounds in all instances. An Excel®-based spectral overlay system was therefore developed. It determined how closely matched the spectral features of the unknown around the respective \( \lambda \) value(s) were to those observed in potential matches identified in the sequence of prior database filtering steps. Comparisons were based on normalised spectra where the absorbance at the \( \lambda \) max of either the unknown or standard was set to 1 to facilitate the comparison of the inflections of the waves. The normalisation was performed using the following formula:

\[
\text{NormalisedABSofstandard/unknown} = \frac{\text{ABSofstandard/unknown}}{\text{ABS} \text{ABS of } \lambda \text{max}}
\]

where \( \text{ABS} \)—absorbance of either the standard or the unknown, \( \text{ABS of } \lambda \text{max} \)—absorbance of the \( \lambda \) maxima/um of either the standard or the unknown.

Based on the normalised absorbances, a threshold of ± 0.125 absorbance units for a potential match was set, and the percentage of absorbance points of the respective honey band that fell within the threshold was determined; the standard that yielded the highest percentage value was considered as a true potential match. To further refine the selection, Pearson’s correlation was also used to determine the relationship of the spectra of the unknown and the standards. If there were multiple candidates, the one with the highest correlation was deemed a true potential match, but only if the difference of the correlation of the matches was found to be greater than ± 0.100. Furthermore, the difference in thresholds when comparing the percent of points of the unknown that fell within that of a potential match was set at ± 10%.

Three spectral overlays were used in the matching process, namely, UV-Vis spectra after development, and also after derivatisation with NP-PEG and with VS reagents. Absorbances within the following wavelength ranges were considered important: 250 to 500 nm for UV-Vis prior to derivatisation and also after spraying with NP-PEG reagent, and 230 to 600 nm for UV-Vis after spraying with VSA reagent. The spectral overlays of fluorescence prior to and after derivatisation with NP-PEG and VSA reagent were only conducted if the UV-Vis spectra were not able to clearly identify the identity of the compound and region; 210 to 270 nm was considered as an important region for all fluorescence spectra.

Spectral matching was performed in the following sequence: UV-Vis prior to derivatisation, UV-Vis after derivatisation with NP-PEG reagent, UV-Vis after derivatisation with VS reagent and then fluorescence prior to and after any derivatisation (optional). A visual inspection of the spectral overlays was also conducted to confirm the match.

4.10. Database Testing and Validation

In order to determine whether the established database and developed filtering algorithm, including spectral overlays, were capable of correctly identifying an unknown compound, positive and negative control samples were analysed, where the positive controls were two artificial honeys spiked with two standards, respectively, which were included in the database to see if they could be correctly identified, and the negative control sample was an artificial honey spiked with a compound not included in the database, to confirm that the screening process would not yield any false-positive identification.
4.11. Inter-Method Validation

To further validate the detection of compounds using the HPTLC-derived databases, blinded test samples were also analysed in a separate laboratory using HPLC-DAD (unpublished protocol).

5. Conclusions

This study reported on a validated HPTLC-derived database system for phenolic compound determination in honey. Extensive research on the reported phenolic compounds in honey was performed prior to the database development to ensure that a comprehensive number of relevant standards could be included in the database. Two pairs of databases were developed that captured Rf values, colour (H◦) at 254 nm and 366 nm, at 366 nm after derivatising with NP-PEG reagent, and at 366 nm, and white light in transmittance mode after derivatising with VSA reagent as well as fluorescence λ max and λ min and UV-Vis λ max after development, and fluorescence and UV-Vis λ max after derivatisation. These were all used as filtering variables to determine compound matches for an unknown sample against the database standards. An automated spectral overlay system was also developed to confirm that the database match correctly portrayed the identity of the unknown compounds. Validation with positive and negative controls in the form of artificial honey spiked with test compounds that were either present or absent from the database as well as the inter-method and inter-lab validations against HPLC-DAD analysis confirmed the reliability of the matching process.

The usefulness of the database was demonstrated by identifying a number of Manuka honey constituents. Leptosperine, mandelic acid, kojic acid, lepteridine, lumichrome, epigallocatechin gallate (EGCG), methyl syringate, gallic acid, mandelic acid, 2,3,4-trihydroxybenzoic acid, o-anisic acid and salicylic acid were identified using the matching strategy against the developed databases.

While the database system was demonstrated in this study to be a powerful tool in identifying unknown compounds in honey, the concept of using rich HPTLC data, not only including Rf values but also colour hues and UV and fluorescence spectra prior to and after derivatisation, can be assumed to also be useful in the identification of other natural product constituents, even in complex matrices, as long as meaningful reference standards can be identified for the construction of the database.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/molecules27196651/s1, Figure S1: Basic Flavone Structure, Figure S2: Basic Flavonol Structure, Figure S3: Basic Flavanone Structure, Figure S4: Basic Flavanonol Structure, Figure S5: Basic Flavan-3-ol Structure, Figure S6: Basic Isoflavonoid Structure, Figure S7: Chalcone, Figure S8: Hydroxybenzoic Acid and its Derivatives (HBADs), Figure S9: Ellagic Acid (42), Figure S10: Hydroxycinnamic Acid and its Derivatives (HCADs), Figure S11: Hydroxyphenylacetic Acid and Derivatives (HPAAD), Figure S12: Hydroxyphenyllactic Acid and Derivatives (HPLAD) and Hydroxyphenylpropanoic Acid and Derivatives (HPPAD), Figure S13: Other phenolic compounds, Figure S14: Dibenzyl oxalate, Figure S15: Structure of the non-phenolic compounds, Figure S16: (A–C) UV-Vis spectra (prior to derivatisisation) overlay of unknown B vs. isorhamnetin and kaempferol (A) and UV-Vis (prior to derivatisisation) spectra overlay of unknown A vs. the ±0.125 AU of isorhamnetin (B) and vs. ±0.125 AU of kaempferol (C), Figure S17: (A–C). UV-Vis spectra (after derivatisisation with NP-PEG reagent) overlay of unknown B vs. isorhamnetin and kaempferol (A) and UV-Vis (after derivatisisation with NP-PEG reagent) spectra overlay of unknown A vs. the ±0.125 AU of isorhamnetin (B) and vs. ±0.125 AU of kaempferol (C), Figure S18: (A–C). UV-Vis spectra (after derivatisisation with VSA reagent) overlay of unknown B vs. isorhamnetin and kaempferol (A) and UV-Vis (after derivatisisation with VSA reagent) spectra overlay of unknown A vs. the ±0.125 AU of isorhamnetin (B) and vs. ±0.125 AU of kaempferol (C), Figure S19: (A–G). UV-Vis spectra (prior to derivatisisation) overlay of unknown C vs. the match compounds and UV-Vis (prior to derivatisisation) spectra overlay of unknown A vs. the ±0.125 AU of hesperitin (B), vs. ±0.125 AU of naringenin (C), vs. ±0.125 AU of benzoic acid (D), ±0.125 AU of m-toluic acid (E), ±0.125 AU of m-coumaric acid (F), and ±0.125 AU of p-MPLA (G), Figure S20: (A–D). UV-Vis spectra (after derivatisisation with
NP-PEG reagent) overlay of unknown C vs. benzoic acid (B), m-toluic acid (C), and 4-MPLA (D) and UV-Vis (after development) spectra overlay of unknown A vs. the ±0.125 AU of benzoic acid (B), ±0.125 AU of m-toluic acid (C), and ±0.125 AU of p-MPLA (D), Table S1: Flavones Standards Used in the Database Development, Table S2: Flavonol Standards Used in the Database Development, Table S3: Flavanone Standards Used in the Database Development, Table S4: Flavanone Standards Used in the Database Development, Table S5: Flavan-3-ol Standards Used in the Database Development, Table S6: Isoflavonoid Standards used in the Database Development, Table S7: t-Chalcone Standard Used in the Database Development, Table S8: Hydroxybenzoic Acid and its Derivatives (HBADs) Standards used in the Database Development, Table S9: Hydroxybenzylc Acid and its Derivatives (HCADs) Standards used in the Database Development, Table S10: Hydroxyphenylacetic Acid and Derivatives (HPAAD) Standards used in the Database Development, Table S11: Hydroxyphenyllactic Acid and Derivatives (HPLAD) and Hydroxyphenylpropanoic Acid and Derivatives (HPPAD) Standards used in the Database Development, Table S12: Other/Miscellaneous Phenolic Standards used in the Database Development, Table S13: Oxalate ester Standard used in the Database Development, Table S14: Non-phenolic compounds used in the Database Development, Table S15: Summary of the data used to determine the identity of the unknown bands in Manuka honey (Database 1A), Table S16: Summary of the data used to determine the identity of the unknown bands in Manuka honey (Database 1B), Table S17: Summary of the data used to determine the identity of the unknown bands in Manuka honey (Database 2A), Table S18: Summary of the data used to determine the identity of the unknown bands in Manuka honey (Database 2B).

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**Sample Availability:** Samples of the compounds and honeys are available from the authors.

**References**

1. Badjah Hadj Ahmed, A.Y.; Wabaidur, S.M.; Siddiqui, M.R.; Alothman, Z.A.; Obeid, M.S.; Khan, M.R.; Al-Tamrah, S.A. Simultaneous determination of twenty-five polyphenols in multifloral and cactus honeys using solid-phase extraction and high-performance liquid chromatography with photodiode array detection. *Eur. Food Res. Technol.* 2016, 242, 943–952. [CrossRef]

2. Lamien-Meda, A.; Lamien, C.E.; Millogo, J.F.; Romito, M.; Nacoulma, O.G. Physicochemical analyses of Burkina Fasan honey. *Acta Vet. Brno* 2005, 74, 147–152. [CrossRef]

3. Zhao, L.; Ren, C.; Xue, X.; Lu, H.; Wang, K.; Wu, L. Safflomin A: A novel chemical marker for *Carthamus tinctorius* L. (Safflower) monofloral honey. *Food Chem.* 2022, 366, 130584. [CrossRef]
4. Das, A.; Datta, S.; Mukherjee, S.; Bose, S.; Ghosh, S.; Dhar, P. Evaluation of antioxidative, antibacterial and probiotic growth stimulatory activities of *Sesamum indicum* honey containing phenolic compounds and lignans. *LWT—Food Sci. Technol.* 2015, 61, 244–250. [CrossRef]

5. Guzelmeric, E.; Ciftci, I.; Yuksel, P.I.; Yesilada, E. Importance of chromatographic and spectrophotometric methods in determining authenticity, classification and bioactivity of honey. *LWT 2020*, 132, 109921. [CrossRef]

6. Nascimento, K.S.D.; Gasparotto Sattler, J.A.; Lauer Macedo, L.E.; Serna González, C.V.; Pereira de Melo, L.L.; da Silva Araújo, E.; Granato, D.; Sattler, A.; de Almeida-Muradian, L.B. Phenolic compounds, antioxidant capacity and physicochemical properties of Brazilian *Apis mellifera* honeys. *LWT—Food Sci. Technol.* 2018, 91, 85–94. [CrossRef]

7. Nguyen, H.T.L.; Panyoyai, N.; Kasapis, S.; Pang, E.; Mantri, N. Honey and Its Role in Relieving Multiple Facets of Atherosclerosis. *Nutrients* 2019, 11, 167. [CrossRef]

8. Stanek, N.; Teper, D.; Kafarski, P.; Jasicka-Misiak, I. HPTLC Phenolic Profiles as Useful Tools for the Authentication of Honey. *J. Agric. Food Chem.* 2019, 67, 199–207. [CrossRef]

9. Sant’anã, L.D.; Buarque Ferreira, A.B.; Lorenzon, M.C.A.; Berbara, R.L.L.; Castro, R.N. Correlation of Total Phenolic and Flavonoid Contents of Brazilian Honeys with Colour and Antioxidant Capacity. *Int. J. Food Prop. 2014*, 17, 65–76. [CrossRef]

10. Michalkiewicz, A.; Biesaga, M.; Pyrzynska, K. Solid-phase extraction procedure for determination of phenolic acids and some flavonoids in honey. *J. Chromatogr. A 2008*, 1187, 18–24. [CrossRef] [PubMed]

11. Gheldof, N.; Wang, X.-H.; Engeseth, N.J. Identification and Quantification of Antioxidant Components of Honeys from Various Floral Sources. *J. Agric. Food Chem.* 2002, 50, 5870–5877. [CrossRef] [PubMed]

12. Demir Kanbur, E.; Yuksek, T.; Atamov, V.; Ozcelik, A.E. A comparison of the physicochemical properties of chestnut and highland honey: The case of Senoz Valley in the Rize province of Turkey. *Food Chem.* 2021, 345, 128864. [CrossRef] [PubMed]

13. Martos, I.; Ferreres, F.; Tomás-Barberán, F.A. Identification of flavonoid markers for the botanical origin of Eucalyptus honey. *J. Agric. Food Chem.* 2000, 48, 1498–1502. [CrossRef] [PubMed]

14. Tomás-Barberán, F.A.; Martos, I.; Ferreres, F.; Radovic, B.S.; Anklam, E. HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. *J. Sci. Food Agric. 2001*, 81, 485–496. [CrossRef]

15. Escriche, I.; Kadar, M.; Juan-Borrás, M.; Domenech, E. Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment. *Food Chem. 2014*, 142, 135–143. [CrossRef]

16. Lawag, I.L.; Lim, L.-Y.; Joshi, R.; Hammer, K.A.; Locher, C. A Comprehensive Survey of Phenolic Constituents Reported in Monofloral Honeys around the Globe. *Food Educ. Leadersh.* 2022, 11, 1152. [CrossRef] [PubMed]

17. Trifković, J.; Andrić, F.; Ristivojević, P.; Guzelmeric, E.; Yesilada, E. Analytical methods in tracing honey authenticity. *J. AOAC Int.* 2017, 100, 827–839. [CrossRef]

18. Green, K.J.; Lawag, I.L.; Locher, C.; Hammer, K.A. Correlation of the antibacterial activity of commercial manuka and *Leptospermum* honeys from Australia and New Zealand with methylglyoxal content and other physicochemical characteristics. *PLoS ONE 2022*, 17, e0272376. [CrossRef]

19. Rückriemen, J.; Henle, T. Pilot study on the discrimination of commercial *Leptospermum* honeys from New Zealand and Australia by HPLC–MS/MS analysis. *Eur. Food Res. Technol.* 2018, 244, 1203–1209. [CrossRef]

20. Stanek, N.; Jasicka-Misiak, I. HPTLC Phenolic Profiles as Useful Tools for the Authentication of Honey. *Food Anal. Methods 2018*, 11, 2979–2989. [CrossRef]

21. Cheung, Y.; Meenu, M.; Yu, X.; Xu, B. Phenolic acids and flavonoids profiles of commercial honey from different floral sources and geographic sources. *Int. J. Food Prop. 2019*, 22, 290–308. [CrossRef]

22. Deng, J.; Liu, R.; Lu, Q.; Hao, P.; Xu, A.; Zhang, J.; Tan, J. Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey. *Food Chem.* 2018, 252, 243–249. [CrossRef]

23. Habib, H.M.; Al Meqbali, F.T.; Camacho, H.; Souka, U.D.; Ibrahim, W.H. Physicochemical and biochemical properties of honeys from arid regions. *Food Anal. Methods 2014*, 71, 35–43. [CrossRef] [PubMed]

24. Hemppattarasuwon, P.; Settachaimongkon, S.; Dangmal, K. Impact of botanical source and processing conditions on physicochemical properties and honey in the northern part of Thailand. *Int. J. Food Sci. Technol.* 2019, 54, 3185–3195. [CrossRef]

25. Combarros-Fuertes, P.; Estevinho, L.M.; Dias, I.G.; Castro, J.M.; Tomás-Barberán, F.A.; Tornadijo, M.E.; Fresno-Baro, J.M. Bioactive Components and Antioxidant and Antibacterial Activities of Different Varieties of Honey: A Screening Prior to Clinical Application. *J. Agric. Food Chem.* 2019, 67, 688–698. [CrossRef]

26. Bong, J.; Loomes, K.M.; Lin, B.; Stephens, J.M. New approach: Chemical and fluorescence profiling of NZ honeys. *Food Chem.* 2018, 267, 355–367. [CrossRef] [PubMed]

27. Lin, B.; Daniels, B.J.; Middleditch, M.J.; Furkert, D.P.; Brimble, M.A.; Bong, J.; Stephens, J.M.; Loomes, K.M. Utility of the *Leptospermum scoparium* Compound Lepteridine as a Chemical Marker for Manuka Honey Authenticity. *ACS Omega 2020*, 5, 8858–8866. [CrossRef]

28. Weston, R.J.; Brocklebank, L.K.; Lu, Y. Identification and quantitative levels of antibacterial components of some New Zealand honeys. *Food Chem.* 2000, 70, 427–435. [CrossRef]

29. Oelschlaegel, S.; Gruner, M.; Wang, P.-N.; Boettcher, A.; Koelling-Speer, I.; Speer, K. Classification and characterization of manuka honeys based on phenolic compounds and methylglyoxal. *J. Agric. Food Chem.* 2012, 60, 7229–7237. [CrossRef] [PubMed]
30. Yao, L.; Bhandari, B.R.; Datta, N.; Singanusong, R.; D’Arcy, B.R. Crystallisation and moisture sorption properties of selected Australian unifloral honeys. *J. Sci. Food Agric.* **2003**, *83*, 884–888. [CrossRef]

31. Anand, S.; Deighton, M.; Livanos, G.; Morrison, P.D.; Pang, E.C.K.; Mantri, N. Antimicrobial Activity of Agastache Honey and Characterization of Its Comparative Composition in Comparison with Important Commercial Honeys. *Front. Microbiol.* **2019**, *10*, 263. [CrossRef] [PubMed]

32. Shen, S.; Wang, J.; Zhuo, Q.; Chen, X.; Liu, T.; Zhang, S.-Q. Quantitative and discriminative evaluation of contents of phenolic and flavonoid and antioxidant competence for Chinese honeys from different botanical origins. *Molecules* **2018**, *23*, 1110. [CrossRef]

33. Stephens, J.M.; Schlothauer, R.C.; Morris, B.D.; Yang, D.; Fearnley, L.; Greenwood, D.R.; Loomes, K.M. Phenolic compounds and methyglyoxal in some New Zealand manuka and kanuka honeys. *Food Chem.* **2010**, *120*, 78–86. [CrossRef]

34. Marshall, S.M.; Schneider, K.R.; Cisneros, K.V.; Gu, L. Determination of antioxidant capacities, α,ω-dicarboxyls, and phenolic phytochemicals in florida varietal honeys using HPLC-DAD-ESI-MSn. *J. Agric. Food Chem.* **2014**, *62*, 8623–8631. [CrossRef]

35. Stephens, J.M.; Loomes, K.M.; Braggins, T.J.; Bong, J.; Lin, B.; Prijic, G. Fluorescence: A Novel Method for Determining Manuka Honey Floral Purity. In *Honey Analysis*; Books on Demand: Norderstedt, Germany, 2017.

36. Islam, M.K.; Sostaric, T.; Lim, L.Y.; Hammer, K.; Locher, C. Development of an HPTLC-based dynamic reference standard for the analysis of complex natural products using Jarrah honey as test sample. *PLoS ONE* **2021**, *16*, e0254857. [CrossRef]

37. Islam, M.K.; Lawag, I.L.; Green, K.J.; Sostaric, T.; Hammer, K.A.; Lim, L.Y.; Locher, C. An investigation of the suitability of melissopalynology to authenticate Jarrah honey. *Curr. Res. Food Sci.* **2022**, *5*, 506–514. [CrossRef]

38. Green, K.J.; Islam, M.K.; Lawag, I.; Locher, C.; Hammer, K.A. Honeys derived from plants of the coastal sandplains of Western Australia: Antibacterial and antioxidant activity, and other characteristics. *J. Apic. Res.* **2022**, *1–14. [CrossRef]

39. Matteini, P.; Agati, G.; Pinelli, P.; Gotti, A. Modes of complexation of rutin with the flavonoid reagent diphenylborinic acid 2-aminoethyl ester. *Mon. Chem.—Chem. Mon.* **2011**, *142*, 885. [CrossRef]

40. Bernardi, T.; Bortolini, O.; Massi, A.; Sacchetti, G.; Tacchini, M.; De Risi, C. Exploring the Synergy Between HPTLC and HPLC-DAD for the Investigation of Wine-Making By-Products. *Molecules* **2019**, *24*, 3416. [CrossRef]

41. Eike Reich, A.S. High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants, 1st ed.; Thieme Medical Publishers, Inc.: New York, NY, USA, 2006; p. 280.

42. Lin, B.; Loomes, K.M.; Prijic, G.; Schlothauer, R.; Stephens, J.M. Lepteridine as a unique fluorescent marker for the authentication of manuka honey. *Food Chem.* **2017**, *225*, 175–180. [CrossRef]

43. Afrin, S.; Giampietri, F.; Forbes-Hernández, T.Y.; Gasparini, M.; Amici, A.; Cianciosi, D.; Quiles, J.L.; Battino, M. Manuka honey synergistically enhances the chemopreventive effect of 5-fluorouracil on human colon cancer cells by inducing oxidative stress and apoptosis, altering metabolic phenotypes and suppressing metastasis ability. *Free Radic. Biol. Med.* **2018**, *126*, 41–54. [CrossRef]

44. Beilich, N.; Köhling-Speer, I.; Oelschlägel, S.; Speer, K. Differentiation of manuka honey from kanuka honey and from jelly bush honey using HS-SPME-GC/MS and UHPLC-PDA-MS/MS. *J. Agric. Food Chem.* **2014**, *62*, 6435–6444. [CrossRef]

45. Bong, J.; Loomes, K.M.; Schlothauer, R.C.; Stephens, J.M. Fluorescence markers in some New Zealand honeys. *Food Chem.* **2016**, *192*, 1006–1014. [CrossRef] [PubMed]

46. Shen, S.; Wang, J.; Chen, X.; Liu, T.; Zhuo, Q.; Zhang, S.-Q. Evaluation of cellular antioxidant components of honeys using UPLC-MS/MS and HPLC-FLD based on the quantitative composition-activity relationship. *Food Chem.* **2019**, *293*, 169–177. [CrossRef] [PubMed]

47. Afrin, S.; Giampietri, F.; Gasparini, M.; Forbes-Hernández, T.Y.; Cianciosi, D.; Reboredo-Rodriguez, P.; Manna, P.P.; Zhang, J.; Quiles, J.L.; Battino, M. The inhibitory effect of manuka honey on human colon cancer HCT-116 and LoVo cell growth. Part 2: Induction of oxidative stress, alteration of mitochondrial respiration and glycolysis, and suppression of metastatic ability. *Food Funct.* **2018**, *9*, 2158–2170. [CrossRef] [PubMed]

48. Khalil, M.I.; Alam, N.; Moniruzzaman, M.; Sulaiman, S.A.; Gan, S.H. Phenolic Acid Composition and Antioxidant Properties of Malaysian honeys. *J. Food Sci.* **2011**, *76*, C921–C928. [CrossRef] [PubMed]

49. Yao, L.; Datta, N.; Tomás-Barberán, F.A.; Ferreres, F.; Martos, I.; Singanusong, R. Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand *Leptospermum* honeys. *Food Chem.* **2003**, *81*, 159–168. [CrossRef]

50. Stanek, N.; Kafarski, P.; Jasicka-Misiak, I.J. Development of a high performance thin layer chromatography method for the rapid qualification and quantification of phenolic compounds and abscisic acid in honeys. *J. Chromatogr. A* **2019**, *1598*, 209–215. [CrossRef] [PubMed]

51. Islam, M.K.; Sostaric, T.; Lim, L.Y.; Hammer, K.; Locher, C. Antioxidant HPTLC-DPPH Fingerprinting of Honeys and Tracking of Antioxidant Constituents Upon Thermal Exposure. *Foods* **2021**, *10*, 357. [CrossRef] [PubMed]

52. Jasicka-Misiak, I.; Makowicz, E.; Stanek, N. Chromatographic fingerprint, antioxidant activity, and colour characteristic of polish goldenrod (*Solidago virgaurea* L) honey and flower. *Eur. Food Res. Technol.* **2018**, *244*, 1169–1184. [CrossRef]

53. Islam, M.K.; Sostaric, T.; Lim, L.Y.; Hammer, K.; Locher, C. Development and validation of an HPTLC–DPPH assay and its application to the analysis of honey. *JPC—J. Planar Chromatogr.—Mod. TLC* **2020**, *33*, 301–311. [CrossRef]

54. Güzelmıc, E.; Ristivojević, P.; Trifković, J.; Dastan, T.; Yilmaz, O.; Cengiz, O.; Yesilada, E. Authentication of Turkish propolis through HPTLC fingerprints combined with multivariate analysis and palynological data and their comparative antioxidant activity. *J. W&T* **2018**, *87*, 23–32. [CrossRef]
55. Guzelmeric, E.; Vovk, I.; Yesilada, E. Development and validation of an HPTLC method for apigenin 7-O-glucoside in chamomile flowers and its application for fingerprint discrimination of chamomile-like materials. *J. Pharm. Biomed. Anal.* 2015, 107, 108–118. [CrossRef]
56. Rusko, J.; Vainovska, P.; Vilne, B.; Bartkevics, V. Phenolic profiles of raw mono- and polyfloral honeys from Latvia. *J. Food Compost. Anal.* 2021, 98, 103813. [CrossRef]
57. Wu, A.H.; French, D. Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. *Clin. Chim. Acta* 2013, 420, 4–10. [CrossRef] [PubMed]
58. Pascual-Maté, A.; Osés, S.M.; Fernández-Muiño, M.A.; Sancho, M.T. Analysis of Polyphenols in Honey: Extraction, Separation and Quantification Procedures. *Sep. Purif. Rev.* 2017, 47, 142–158. [CrossRef]
59. Sik, B.; Szévelyhidi, R.; Lakatos, E.; Kacsándi, V.; Ajtory, Z. Analytical procedures for determination of phenolics active herbal ingredients in fortified functional foods: An overview. *Eur. Food Res. Technol.* 2022, 248, 329–344. [CrossRef]
60. Neveu, V.; Perez-Jiménez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; et al. Phenol-Explorer: An online comprehensive database on polyphenol contents in foods. *Database* 2010, 2010, bap024. [CrossRef]
61. Ferreira, J.F.; Luthria, D.L.; Sasaki, T.; Heyerick, A. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 2010, 15, 3135–3170. [CrossRef] [PubMed]
62. Jan, S.; Abbas, N. Chapter 4—Chemistry of Himalayan Phytochemicals. In *Himalayan Phytochemicals*; Jan, S., Abbas, N., Eds.; Elsevier: Amsterdam, The Netherlands, 2018; pp. 121–166.
63. Gafner, S.; Bergeron, C.; Batcha, L.L.; Angererhofer, C.K.; Sudberg, S.; Sudberg, É.M.; Guinaudeau, H.; Gauthier, R. Analysis of *Scutellaria lateriflora* and Its Adulterants *Teucrium canadense* and *Teucrium chamaedrys* by LC–UV/MS, TLC, and Digital Photomicroscopy. *J. AOAC Int.* 2003, 86, 453–460. [CrossRef]
64. Locher, C.; Tang, E.; Neumann, J.; Sostaric, T. High-performance thin-layer chromatography profiling of Jarrah and Manuka honeys. *JPC—J. Planar Chromatogr.—Mod. TLC* 2018, 31, 181–189. [CrossRef]
65. Lawag, I.L.; Yoo, O.; Lim, L.Y.; Hammer, K.; Locher, C. Optimisation of Bee Pollen Extraction to Maximise Extractable Antioxidant Constituents. *Antioxidants* 2021, 10, 1113. [CrossRef] [PubMed]
66. Islam, M.K.; Vinsen, K.; Sostaric, T.; Lim, L.Y.; Locher, C. Detection of syrup adulterants in manuka and jarrah honey using HPTLC-multivariate data analysis. *PeerJ* 2021, 9, e12186. [CrossRef] [PubMed]
67. Jesionek, W.; Majer-Dziedzic, B.; Choma, I.M. Separation, Identification, and Investigation of Antioxidant Ability of Plant Extract Components Using TLC, LC–MS, and TLC–DPPH. *J. Liq. Chromatogr. Relat. Technol.* 2015, 38, 1147–1153. [CrossRef]