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# HPLC-MS Identification of the Most Abundant Flavonoid Glycosides in the Pods of *Abelmoschus esculentus* – Comments on the Published Data

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**Abstract :** Okra (*Abelmoschus esculentus* (L) Moench) is a common vegetable cultivated in warm climate regions. It is also a valuable medicinal plant whose potential health benefits are related to the presence of phenolic compounds, e.g. flavonoids. However, the published results concerning the most abundant flavonoids present in okra fruits are often inconsistent or disputable. Therefore, we have decided to perform the HPLC-MS analysis of okra fruit extract in order to identify the most abundant flavonoid glycosides. The structures of the flavonoid glycosides have been mainly elucidated on the basis of *m/z* values and relative abundances of $[M+/-H]^{+/-}$ precursor ions and $Y_{0/1}^{+/-}$, $[Y_0-H]^{-\bullet}$ product ions. We have identified five compounds, quercetin 3-*O*-glucoside and its four conjugates, which is consistent with part of the literature data, as discussed in details.

**Keywords :** okra pods, flavonoids, liquid chromatography, electrospray ionization mass spectrometry, fragmentation pathways

## Introduction

Okra (*Abelmoschus esculentus* (L) Moench) is a common vegetable widely cultivated in warm climate zones. The okra pods, due to their taste and nutritional values, are ingredients of meals all over the world. It is also a valuable medicinal plant showing various therapeutic activities.[1-5]

It is clear that potential health benefits of okra consumption are related to the presence of phenolic compounds, e.g. flavonoids. However, the published data concerning the most abundant flavonoids present in okra are often inconsistent or disputable. Recently, we have commented on the MS identification of phenolic compounds (including flavonoids) by Wang et al.[6] The identification of the disputable compounds has been summarized in Table 5,[7] and as an erratum the authors have published the identical Table.[8]

We have found that the other papers concerning identification of flavonoids in okra are also sometimes slightly inconsistent or a little disputable in some issues, e.g. concerning the compounds identification (e.g. rutin), detected product ions, determined interglucosidic linkage (as discussed in details further in the text). Therefore, we have decided to perform the high pressure liquid chromatography-mass spectrometric analysis (HPLC-MS) of the okra pods extract in order to identify the most abundant flavonoid glycosides.

## Experimental

Commercially available dried okra pods have been obtained from Samira sp. z o.o. Warsaw, Poland (country of origin: Syria). Standards of quercetin, isorhamnetin, quercetin-3-*O*-glucoside and rutin were obtained from Sigma-Aldrich (Poznań, Poland).

A 2 g portion of dried okra pods (*Abelmoschus esculentus* (L) Moench) was extracted with 10 mL of pure methanol, the sample was shaken at 500 rpm for 30 minutes (Vortex 3, IKA-Werke GmbH, Germany), sonicated and filtered through syringe filters with a pore size of 0.45 μm (Macherey-Nagel GmbH, Germany). Prior to the HPLC/ESI-MS analysis, the sample was further diluted at 1:1 in pure methanol (stored at 5°C). In order to perform extraction and partial hydrolysis of the extracted compounds simultaneously, a 5% methanolic solution of hydrochloric acid (30%, ultra-pure, Chem-Lab) was used instead of pure methanol. The further procedure was the same as above.

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The HPLC-MS analyses were performed using a Waters model 2690 HPLC pump, a Waters SQD mass spectrometer (single quadrupole type instrument equipped with electrospray ion source, Z-spray, Milford, MA, USA). The software used was MassLynx V3.5 (Manchester, UK). Using an autosampler, the sample solutions were injected onto the XTerra® MS C18 column (5 μm, 150 mm × 3 mm i.d.). The injection volume was 10 μL. The solutions were analyzed by using linear gradient of $CH_3CN$-$H_2O$ with a flow rate of 0.5 mL/min The gradient started from 0% $CH_3CN$ - 95% $H_2O$ with 5% of a 10% solution of formic acid in water, reaching 95% $CH_3CN$ after 15 min, and the latter concentration was maintained for 10 min. The ESI mass spectra were recorded in the *m/z* range 100-1000, in positive and negative modes simultaneously (during the HPLC-MS analyses the mass spectrometer was switched in the fast mode between the positive and negative ion modes). The ESI source potentials were as follows: capillary, 3 kV; lens, 0.5 V; extractor, 4 V; cone voltage (CV), 30, 50 or 100 V. This parameter has the greatest impact on the full scan mass spectra recorded. An increase in this parameter leads to the so-called "in-source" fragmentation/dissociation. At CV = 30 V there was no fragmentation, at CV = 50 V the fragmentation of $[M+H]^+$ ions occurred, at CV = 100 V the fragmentation of $[M-H]^-$ ions occurred. The source temperature was 120°C and the desolvation temperature was 300°C. Nitrogen was used as the nebulizing and desolvating gas at flow rates of 100 and 300 L $h^{-1}$, respectively.

The HPLC-HRMS analyses were performed in positive ion mode using a UltiMateTM 3000 UHPLC system (ThermoScientific/Dionex) and Impact HD mass spectrometer (QTOF type instrument equipped with electrospray ion source; Bruker Daltonics). Using an autosampler, the sample solutions were injected onto the Kinetex C18 column (100 × 2.10 mm i.d., 2.6 μm particle size; Phenomenex). The used mobile phases were water with 0.1% of formic acid (solvent A) and acetonitrile with 0.1% of formic acid (solvent B). The used gradient is shown in Table 1.

The flow rate was 0.3 mL/min and the column temperature was maintained at 35°C. The instrument was operated under the following optimized settings: end plate voltage 500 V, capillary voltage 3.6 kV; nebulizer pressure 1.5 bar; dry gas (nitrogen) temperature 200°C; dry gas flow rate 8 L/min. The spectrometer was previously calibrated with the standard tune mixture.

## Results and Discussion

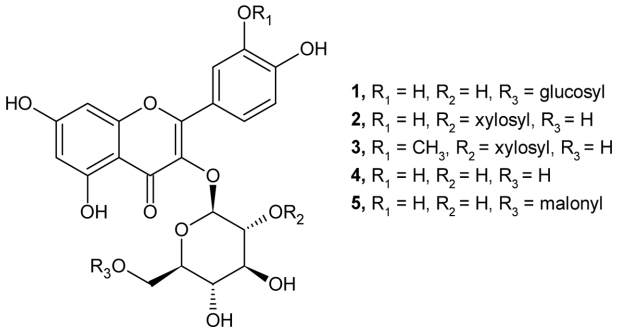
The obtained results of HPLC-MS analysis are summarized in Scheme 1 and Table 1 (exemplary chromatograms and mass spectra are shown in the supplementary material, Figure S1-S7). The identified compounds (1-5) are quercetin 3-*O*-glucoside (isoquercitrin) and its four conjugates.

It has to be stressed that the obtained accurate values of $[M+H]^+$ ions (supplementary material, Figure S7) and *m/z* of the detected product ions (Table 1), as well as the product ion abundances, have fully confirmed the claimed structures. Furthermore, in the acidified extract, due to the partial hydrolysis of flavonoid glycosides, two free aglycones were identified, namely quercetin and isorhamnetin (Figure S5). Identification of aglycone was confirmed by comparison of its retention times with those of the reference standards, and by observation of characteristic product ions, e.g. at *m/z* 151(-) for quercetin, and at *m/z* 300 ($[M-H-CH_3]^{-\bullet}$ ion) for isorhamnetin.[9] Our results indicate that okra is rich in quercetin-3-*O*-glycosides, which has been confirmed by the presence of $[Y_0]^-$ product ion at *m/z* 301, analogously as in the other findings.[10-12] However, quercetin-3-*O*-glycosides should also yield abundant $[Y_0\text{-}H]^{-\bullet}$ product ion at *m/z* 300,[13-15] which, to the best of our knowledge, has been mentioned only in the paper by Panighel et al.[16] Some of the papers contain data which can be considered as typos that should not have been made. For example, the exact *m/z* of rutin $[Y_0]^-$ product ion is 301.0354, whereas it has been reported the accurate value at *m/z* 301.1387, which means unacceptable error 343 ppm.[12]

The low abundant product ion at *m/z* 463(-) confirmed the (1 → 6) interglucosidic linkage in compound **1**.[17] If compound **1** would contain (1 → 2) interglucosidic linkage (quercetin-3-*O*-sophoroside) the abundant product ion at *m/z* 445(-) should be observed.[18] In most of the papers on okra pods glycosides, the detection of quercetin 3-*O*-gentiobioside ((1 → 6) interglucosidic linkage) has been indicated, whereas there are two papers in which the detection of quercetin-3-*O*-sophoroside ((1 → 2) interglucosidic linkage) has been claimed.[19,20] In the abstract of the paper by Wang et al., there is the name "5,7,3',4'-tetrahydroxyflavonol-3-*O*-[β-D-

**Table 1.** The gradient used for HPLC-HRMS analyses

| Time [min] | A [%] | B [%] |
|---|---|---|
| 0 | 97 | 3 |
| 1 | 97 | 3 |
| 20 | 10 | 90 |
| 23 | 10 | 90 |
| 24 | 97 | 3 |




**1,** $R_1$ = H, $R_2$ = H, $R_3$ = glucosyl
**2,** $R_1$ = H, $R_2$ = xylosyl, $R_3$ = H
**3,** $R_1$ = $CH_3$, $R_2$ = xylosyl, $R_3$ = H
**4,** $R_1$ = H, $R_2$ = H, $R_3$ = H
**5,** $R_1$ = H, $R_2$ = H, $R_3$ = malonyl

**Scheme 1.** Structures of the identified flavonoid glycosides (**1-5**).

**Table 2.** The results of HPLC-MS analysis obtained for flavonoid glycosides (**1-5**).

| Compound | $R_t$ (min) | $[M-H]^-$ $[M+H]^+$ | Product ions (type) |
|---|---|---|---|
| **1**, Quercetin-3-*O*-gentiobioside | 7.7 | 625 | 463 ($Y_1^-$), 301 ($Y_0^-$), 300 ($[Y_0\text{-}H]^{-\bullet}$) |
| | | 627 | 465 ($Y_1^+$), 303 ($Y_0^+$) |
| **2**, Quercetin-3-*O*-sambubioside | 8.0 | 595 | 301 ($Y_0^-$), 300 ($[Y_0\text{-}H]^{-\bullet}$) |
| | | 597 | 465 ($Y_1^+$), 303 ($Y_0^+$) |
| **3**, Isorhamnetin-3-*O*-sambubioside | 8.3 | 609 | 315 ($Y_0^-$), 314 ($[Y_0\text{-}H]^{-\bullet}$), 300 ($[Y_0\text{-}CH_3]^-$) |
| | | 611 | 479 ($Y_1^+$), 317 ($Y_0^+$) |
| **4**, Quercetin-3-*O*-glucoside | 8.5 | 463 | 301 ($Y_0^-$), 300 ($[Y_0\text{-}H]^{-\bullet}$) |
| | | 465 | 303 ($Y_0^+$) |
| **5**, 6''-*O*-Malonylisoquercitrin | 8.7 | 549 | 505 ($[M\text{-}H\text{-}CO_2]^-$), 463 ($[M\text{-}H\text{-}CO_2\text{-}CH_2CO]^-$), 301 ($Y_0^-$), 300 ($[Y_0\text{-}H]^{-\bullet}$) |
| | | 551 | 303 ($Y_0^+$) |

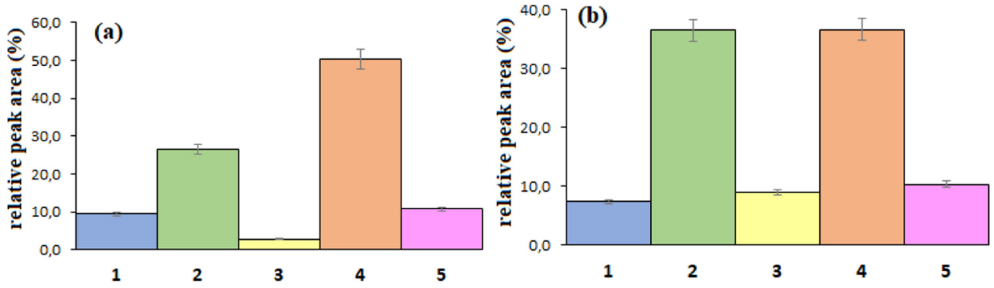



**Figure 1.** Relative chromatographic peak areas of $[M-H]^-$ and $[M+H]^+$ ions obtained in our work (**(a)** and **(b)**, respectively).

rhamnopyranosil-(1→2)]-*β*-D-glucopyranoside", whereas in the supplementary material of the paper by Wang et al., there is a different structure.[19] Compound **1** also cannot be a di-*O*-glucoside, since fragmentation pattern of di-*O*-glycosides, in the negative ion mode, is completely different than that of *O*-diglycosides.[21]

The evident discrepancy between our results and a number of published results is the lack of rutin in our extract (of course its presence in very low amount cannot be excluded), since identification of rutin has been claimed in many papers.[11,12,22-26] On the other hand, we have identified isorhamnetin glycoside, namely isorhamnetin-3-*O*-sambubioside, which has an identical nominal molecular mass as rutin, 610 amu (but of course rutin and isorhamnetin-3-*O*-sambubioside have completely different fragmentation patterns and slightly different retention times, Figures S1, S6). There are a few papers in which the presence of isorhamnetin glycosides has been claimed in okra pods,[7,10,27] however, to the best of our knowledge, there is no paper in which the identification of both rutin and isorhamnetin glycoside has been claimed in one extract of okra pods. Wang et al. have identified isorhamnetin-3-*O*-glucoside,[7] Zhang et al. have detected isorhamnetin glycoside (e.g. "isorhamnetin 3-*O*-glucose-7-*O*-xyloside") in the pods of all 15 okra cultivars studied,[27] and Arapitsas have identified "isorhamn 3-*O*-glu-pent" and this result is in full agreement with our result.[10] Whereas there is a number of papers reporting that isorhamnetin conjugates have not been detected in okra pods, e.g. that by Huang et al., which have not detected isorhamnetin in the hydrolysed extracts of okra pods collected from three different locations.[28] Furthermore, Arapitsas has found five flavonoid glycosides in okra skin, which have been identical to those found in our finding. Yang et al., who reported similar results to ours, found six flavonoid glycosides in okra pods.[11] Among them, the least abundant (1% of the total amount) has been quercetin-7-*O*-glucoside for which the authors have detected only $[M-H]^-$ ion (no product ions), therefore its identification can be questioned. Among the other compounds, Yang et al. have claimed the rutin detection. Assuming that rutin has been wrongly identified instead of isorhamnetin-3-*O*-sambubioside (e.g. due to the identical molecular masses of these compounds), the compounds identified by Yang et al., would be the same as those in our work (Scheme 1). It is also worth mentioning that isorhamnetin-3-*O*-glucoside has the identical molecular mass as very rare quercetin-3-*O*-

(4′′-*O*-methyl)-glucoside (they are isomers) claimed to be present in okra fruit.[29-31] Therefore, it is possible that isorhamnetin-3-*O*-glucoside has been mistaken for this rare compound. It is worth adding that isorhamnetin-3-*O*-glucoside has been unambiguously identified in okra leaves.[16]

Taking into account the similarities between our results and those obtained by Arapitsas for okra skin and by Yang et al. for okra pods,[10,12] we have compared the chromatographic peak areas of $[M+H]^+$ and $[M-H]^-$ ions obtained in our work (they approximately reflect the relative abundances of **1-5**) with the abundances of compounds **1-5** determined by Arapitsas and by Yang et al.[10,12] Figure 1 shows the calculated relative peak areas of **1-5** (expressed as a percentage of the total peak area). The calculated relative abundances of **1-5** (expressed as a percent of the total amount of **1-5**) determined by Arapitsas are 29, 22, 4, 37 and those by Yang et al. are 52, 11, 2, 27, and 7 , respectively. The only evident difference between our results and those obtained by Arapitsas and by Yang et al. is the relatively low abundance of quercetin-3-*O*-gentiobioside (**1**) found in our work in comparison with its high abundance in the works by Arapitsas and by Yang et al.[10,12] On the other hand, the relative abundances of compounds **2-5** can be regarded as comparable.

## Conclusions

Taking into account the consistency between our results and those obtained by Arapitsas,[10] by Yang et al.,[12] as well as by Zhang et al.,[27] it is reasonable to suppose that compounds **1-5** can be regarded as the most abundant flavonoid glycosides present in okra pods, independently of okra origin. Since beside 3, the identified compounds are quercetin glycosides, okra pods can be regarded as a rich source of quercetin, an important flavonol which is a common ingredient of nutraceutical and cosmeceutical products and which has found a number of medicinal applications.[32] Of course, the occurrence of other flavonols cannot be excluded, e.g. three of the 15 okra varieties tested by Zhang et al., were found to contain low amount of kaempferol and myricetin glycosides, therefore, the occurrence of kaempferol and myricetin glycosides in okra pods is also absolutely possible.[27] Furthermore, some structural changes depending on okra cultivar also cannot be excluded e.g. 6"-*O*-acetylisoquercitrin instead of 6"-*O*-malonylisoquercitrin (the former has been found in okra seeds,[33]).

## Conflicts of interest

Authors declare no conflicts of interest.

## Acknowledgements

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## Notes

[†]Electronic Supplementary Information (ESI) available: supplementary material contains exemplary chromatograms and mass spectra, Figure S1-S7, See DOI: 10.5478/MSL.2025.16.3.73

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