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Quantitative Characterization of Flavonoid Compounds in Rooibos Tea (*Aspalathus linearis*) by LC−UV/DAD

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Rooibos tea originates from the leaves and stems of the indigenous South African plant *Aspalathus linearis*. It has gained much attention for clinical purposes in the case of nervous tension, allergies (dermatitis), and various indigestive problems. Recently, antioxidative activity was also attributed to the tea on the basis of its flavonoid content. Therefore, an HPLC method using a C_{18} reversed phase column was developed for the assay of 10 flavonoids in aqueous and methanolic infusions. Main compounds determined were the dihydrochalcone aspalthin, rutin, and orientin, and their content was in the range of 1.0 to 1.3 mg/g. The identity of detected flavonoids was confirmed by comparing their retention times and UV and MS spectra with those of corresponding standards. In addition, the MS analysis showed evidence of the presence of other compounds such as nothofagin, dihydroisoorientin, and dihydroorientin.

KEYWORDS: Rooibos tea; *Aspalathus linearis*; flavonoids; HPLC−UV; LC−MS

INTRODUCTION

Rooibos (*Aspalathus linearis*) is a leguminous shrub native to the mountainous areas of the northwestern Cape Province in South Africa (*1*). The Rooibos plant is recognized as one of the relatively few economic plants that has made the transition from a local wild resource to a cultivated crop in the 20th century. Its leaves and fine stems are used for the production of Rooibos tea; the leaves and stems are cut to 3−4 mm lengths, rolled, fermented by leaf enzymes, and dried in the sun (*2*). Rooibos tea is a beverage rich in volatile compounds (*3*), polyphenols, and minerals. Differently from *Camellia sinensis* tea, Rooibos tea is caffeine-free and has a low tannin content (as gallic acid). Because of these characteristics, Rooibos tea is rapidly gaining in popularity as a health beverage.

Previous research (*4*−*7*) on the presence of flavonoid compounds in Rooibos tea revealed the occurrence of the flavonol quercetin and its 3-*O*-*β*-D-glucopyranoside derivative (isoquercitrin) and the quercetin-3-*O*-rutinoside (rutin). Then, the presence of the aglycons luteolin and chrysoeriol, the dihydrochalcones aspalathin and nothofagin, and the flavones orientin, iso-orientin, and their 4′-deoxy analogues, vitexin and iso-vitexin, has also been described. Catechin, procyanidin B3, and a profistinidin triflavanoid also have been identified. However, the contents of these three compounds are extremely low, and underline the claim that Rooibos tea has a low tannin content (*4*, *7*). The phenolic acids present in Rooibos tea are caffeic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, and protocatechuic acid (*7*), and syringic acid (*4*).

Table 1. Gradient Elution[a] Employed for Reversed-Phase HPLC Separation of Flavonoids in Rooibos Tea

time (min)	solvent composition (% acetonitrile)[b]
0	5
5	25
13	25
20	50
25	80
30	5

[a] 0.8 mL min^{-1}. [b] Gradient of acetic acid–water (0.1%, v/v) and acetonitrile.

Table 2. Detected Ions for Flavonoid Standards

standard	RT (min)	MW (Da)	$[M-H]^-$ (*m*/*z*)	$[M+H]^+$ (*m*/*z*)	$[M+Na]^+$ (*m*/*z*)
isoorientin	11.2	448.36	446.9	449.0	471.0
orientin	11.5	448.36	446.9	449.0	(471.0)
aspalathin	11.9	452.48	451.0	n.d.[a]	474.0
vitexin	12.5	432.36	431.0	432.8	(454.7)
rutin	13.3	610.51	609.3	n.d.	632.3
isovitexin	13.8	432.36	431.0	432.8	454.7
isoquercitrin	15.9	464.37	463.0	n.d.	486.5
luteolin	25.6	286.2	284.9	287.3	n.d
quercetin	25.9	302.23	300.8	303.3	n.d
chrysoeriol	27.0	300.2	299.0	301.2	n.d

[a] Non-detectable.

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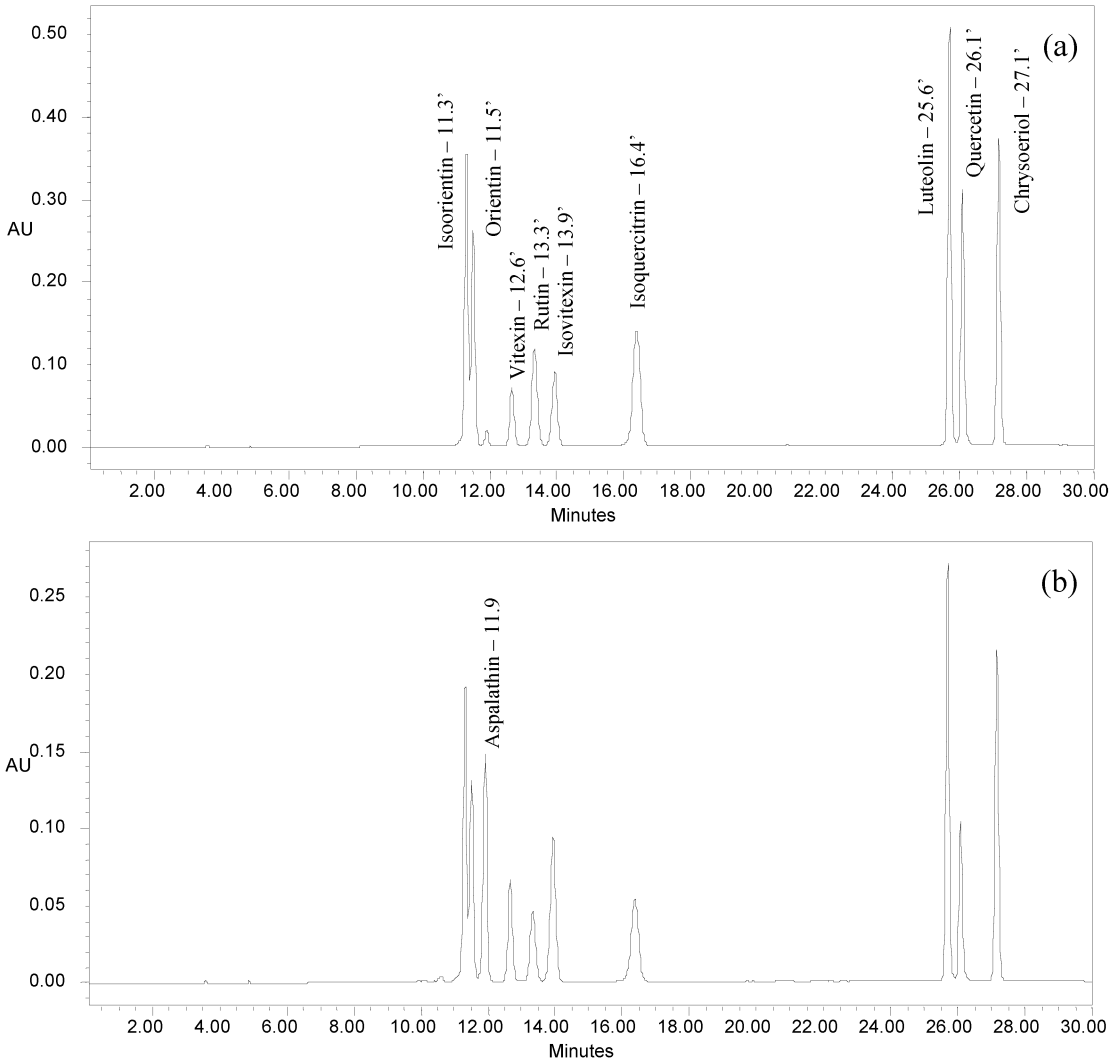

Figure 1. Typical HPLC chromatogram of 10 flavonoid standards, $c = 1$ μg/mL, injected volume 50 μL, at $\lambda = 255$ nm (a) and 287 nm (b).

Table 3. Equations of Calibration Curves Obtained for Flavonoid Standards

standard	calibration curves	R^2
isoorientin	$Y = 179099{,}073 + 3073{,}084$	0.999
orientin	$Y = 125001{,}786 + 1175{,}520$	0.998
aspalathin	$Y = 113668{,}787 - 2004{,}995$	0.999
vitexin	$Y = 66792{,}599 - 129{,}368$	0.989
rutin	$Y = 136806{,}730 + 245{,}240$	0.999
isovitexin	$Y = 105174{,}666 - 397{,}132$	0.998
isoquercitrin	$Y = 224616{,}167 - 1914{,}066$	0.995
luteolin	$Y = 376396{,}119 - 1035{,}932$	0.989
quercetin	$Y = 234740{,}899 - 9110{,}757$	0.988
chrysoeriol	$Y = 269261{,}933 + 241{,}860$	0.999

Table 4. Determination of Flavonoid Compounds in Aqueous and Methanolic Extract (mg/g ± SD)

compound	aqueous extract	methanolic extract
isoorientin	0.833 ± 0.007	1.116 ± 0.012
orientin	1.003 ± 0.010	1.122 ± 0.014
aspalathin	1.234 ± 0.010	1.442 ± 0.014
vitexin	0.330 ± 0.002	0.385 ± 0.004
rutin	1.269 ± 0.006	1.108 ± 0.002
isovitexin	0.265 ± 0.002	0.323 ± 0.001
isoquercitrin and hyperoside	0.429 ± 0.002	0.518 ± 0.006
luteolin	0.029 ± 0.001	0.159 ± 0.001
quercetin	0.107 ± 0.002	0.418 ± 0.001
chrysoeriol	0.022 ± 0.001	0.110 ±0.001
total	5.521 ± 0.003	6.702 ± 0.023

Processing, including fermentation, has a significant influence on the phenolic composition of Rooibos tea. The dihydrochalcone aspalathin, which is the major flavonoid of unfermented leaves and stems, is oxidized during fermentation (*8*) to dihydroiso-orientin (*6*). Similarly, nothofagin, which is structurally similar to aspalathin, is present in relatively large amounts in unprocessed Rooibos tea, but it also undergoes oxidation during fermentation (*6*).

Rooibos tea is consumed for calming digestive disorders and various stomach problems, for reducing nervous tension, and alleviating allergies (*1*). It is used also for topical treatment of dermatological diseases, such as Behcet's disease and photosensitive dermatitis (*10*).

Rooibos tea infusions have a good antioxidative activity (*11*), which can be attributed to the presence of flavonoids and phenolic acids. Indeed, these compounds are known to play an active role in reducing the formation of reactive oxygen species (ROS), because they affect enzymes that catalyze redox reactions and chelate metal ions, such as Cu and Fe, involved in ROS production (*12*). In addition, the highly oxidizing ROS are reduced by flavonoids, which are transformed in less aggressive aroxyl radicals (*13*).

As previously mentioned, different flavonoids have been identified in Rooibos tea including flavonols, flavones, and dihydrochalcones. However, data on the content of these

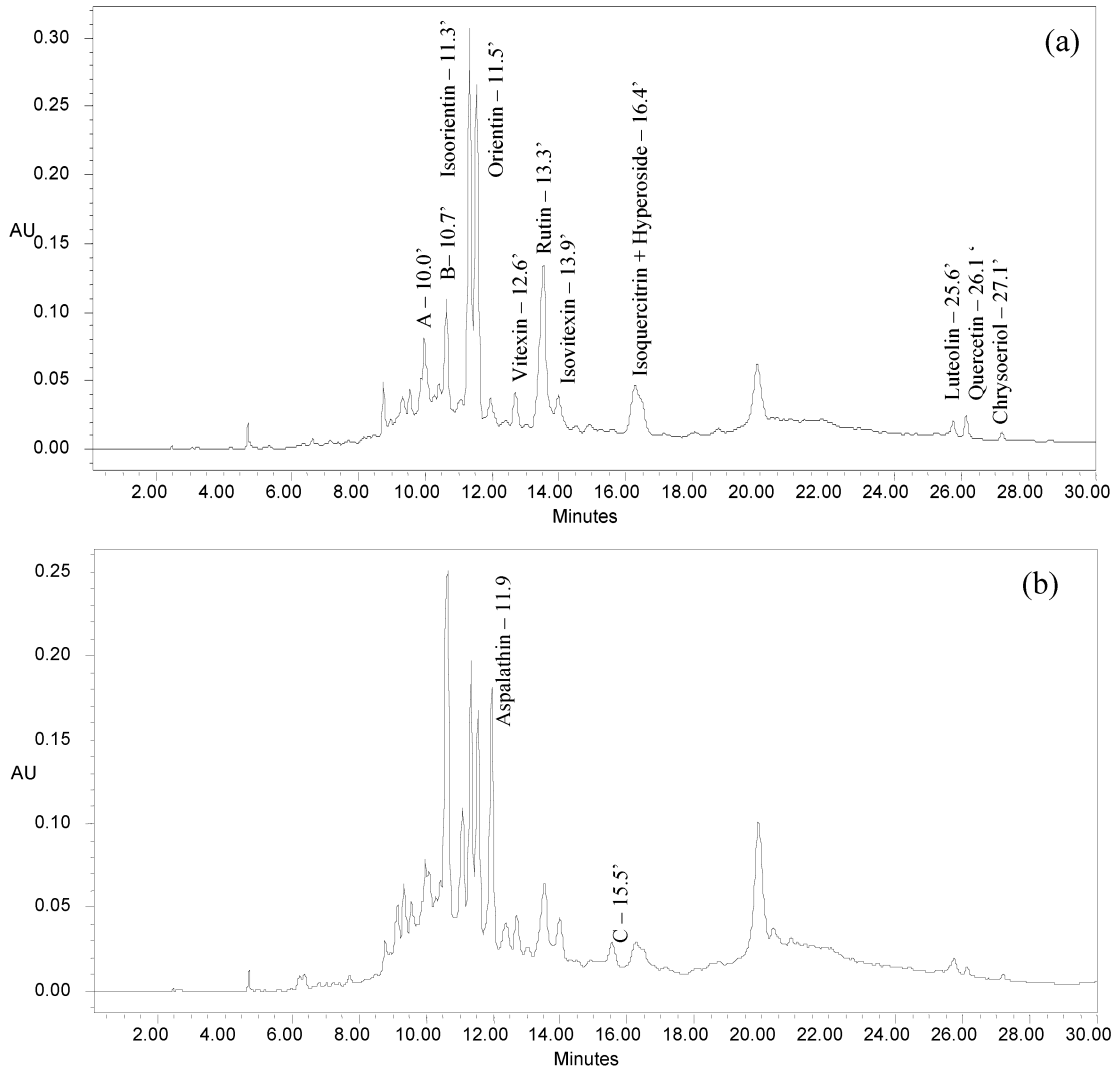

Figure 2. Typical HPLC chromatogram of Rooibos tea aqueous infusion, injected volume 50 μL, at λ = 255 nm (a) and 287 nm (b).

Table 5. Intra- and Interday Analytical Precision of Ten Flavonoid Standards of *Aspalathus linearis*

peak name	*C* (μg/mL)	intraday RSD (%)	interday RSD (%)
isoorientin	1.0	2.12	2.27
	0.1	2.51	2.48
orientin	1.0	2.42	1.57
	0.1	2.61	2.90
aspalathin	1.0	2.09	2.59
	0.1	2.29	1.16
vitexin	1.0	2.14	1.85
	0.1	2.95	2.38
rutin	1.0	2.36	3.27
	0.1	4.08	1.56
isovitexin	1.0	2.19	2.81
	0.1	3.46	0.83
isoquercitrin	1.0	2.10	2.90
	0.1	3.60	2.27
luteolin	1.0	2.60	4.13
	0.1	2.84	3.21
quercetin	1.0	1.99	4.80
	0.1	6.83	3.37
chrysoeriol	1.0	2.10	1.81
	0.1	1.64	1.57

compounds are lacking. The aim of this study was the quantitative determination of the different flavonoid compounds by LC−UV/DAD extracted either by water or methanol. In addition, mass spectrometric analyses were applied to confirm or suggest peak identity.

MATERIALS AND METHODS

Chemicals. Rutin, orientin, isoorientin, vitexin, isovitexin, isoquercitrin, hyperoside, luteolin, and chrysoeriol were purchased from Extrasynthese (Genay, France). Quercetin was purchased from Sigma-Aldrich (Steinheim, Germany) and aspalathin was from Leuven Bioproducts (Belgium). Methanol and acetonitrile were HPLC grade from Merck (Darmstadt, Germany). Standards were dissolved in methanol (1 mg/mL) and stored at −20 °C. Aliquots of standard solutions in the range of 0.1−20 μg/mL were injected into the HPLC apparatus.

Water and Methanol Extractions of Polyphenol Fraction from Rooibos Tea. Commercial Rooibos tea bags were obtained from Rooibos LTD-BPK (Clanwilliam, South Africa) and from Nutritea (Cedaberg, South Africa).

The extractions were prepared by infusion with either hot water or cold methanol. The aqueous extract was obtained by pouring 60 mL of hot distilled water on 1 g of tea and steeping it for 10 min. The extract was cooled to room temperature, filtered through filter paper, made up to volume (100 mL) with distilled water, and centrifuged at 2000*g* for 10 min. The solution was stored at −20 °C until time of analysis.

The methanolic extract was obtained by infusing 1 g of Rooibos tea in 60 mL of methanol and stirring the suspension for 16 h. The extract was filtered through filter paper, made up to volume (100 mL) with

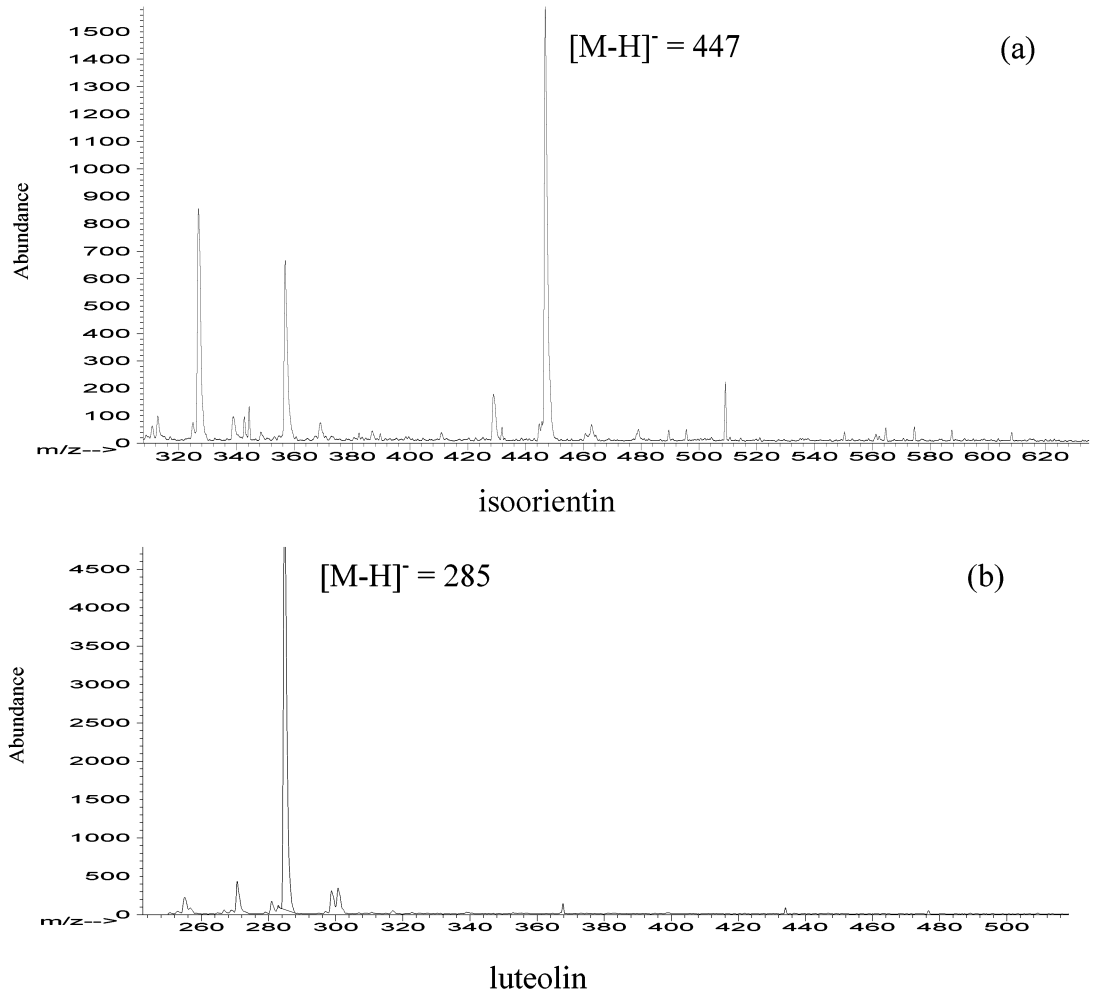

Figure 3. MS online spectra in the negative scan mode of standards of isoorientin (a) and luteolin (b).

methanol, and centrifuged at 2000*g* for 10 min. The solution was stored at −20 °C until time of analysis.

Chromatographic Conditions. HPLC separation was performed using a Waters 625 LC System (Milford, MA) connected with a Waters model 996 photodiode array detector, equipped with a Rheodyne injector (loop 50 μL) and a Millennium workstation (Waters).

The column was a 5 μm Symmetry Shield C_{18} (250 × 4.6 mm i.d.) from Waters. The eluents were (A) 0.1% acetic acid and (B) acetonitrile. Separations were performed at room temperature by solvent gradient elution (**Table 1**) at a flow-rate of 0.8 mL min^{-1}. Acquisition was set at 255 and 287 nm (Spectral acquisition in the range 200−400 nm).

Mass Spectrometry. Peaks were analyzed using a Hewlett-Packard 5989A quadrupole mass spectrometer equipped with an electrospray interface (HP 59987A). Nitrogen was used as nebulizing gas at an inlet pressure of 50 psi and a temperature of 300 °C. ESI-MS conditions were optimized by flow injection of rutin standard solution. Analyses were carried out in negative and positive scan modes from *m*/*z* 150 to 1000.

Capillary Electrophoresis. Capillary electrophoresis separations were performed using an Applied Biosystems 270A instrument, following the analytical method described by Pietta et al. (*14*).

RESULTS AND DISCUSSION

Typical HPLC−UV chromatograms at 255 and 287 nm of a standard mixture are shown in **Figure 1a** and **b**, respectively. The slightly acidified water−acetonitrile gradient was suitable for obtaining well-resolved and symmetrical peak separation for nearly all compounds. Only orientin and iso-orientin resulted incompletely separated in comparison to the other standards, but they could be accurately integrated. Flavonoids extracted from Rooibos tea using water or methanol were identified by comparison of their retention times and UV spectra (at 255 and 287 nm) with those of the reference standards. **Figure 2** shows typical chromatograms of an aqueous infusion of Rooibos tea at 255 (panel **a**) and 287 nm (panel **b**). Similar chromatograms were obtained from the methanolic extracts.

Table 2 summarizes the ions detected for 10 different flavonoid standards. Glycosylated flavonoids were detectable in positive and negative scan modes. In the positive scan mode, the glycosylated flavonoids isoorientin and isovitexin were detected as $[M + H]^+$ ions as well as sodiated adducts ($[M + Na]^+$). By contrast, due to their structural properties, other glycosides such as orientin and vitexin were present mainly as $[M + H]^+$ ions, whereas aspalathin, rutin, and isoquercitrin yielded only sodiated adducts. Negative scan mode permitted the best ion detection ($[M - H]^-$) of the aglycons in standard and tea solution. To exemplify, **Figure 3** shows mass spectra of the glycoside isoorientin and the aglycon luteolin in the negative scan mode.

Mass spectrometry coupled with HPLC permitted confirmation of the nature of 10 flavonoids already described in the literature by comparing their retention times, UV spectra, and MS spectra with those of standards. To exemplify, in **Figure 4** the MS online spectra of isoorientin, aspalathin, rutin, and luteolin obtained from a Rooibos tea infusion are shown. LC−MS analyses also permitted suggested structures of at least 3 other flavonoids; based on their mass spectra, peaks A, B, and C in **Figure 2** may be assigned as dihydro-isoorientin (RT 10.0

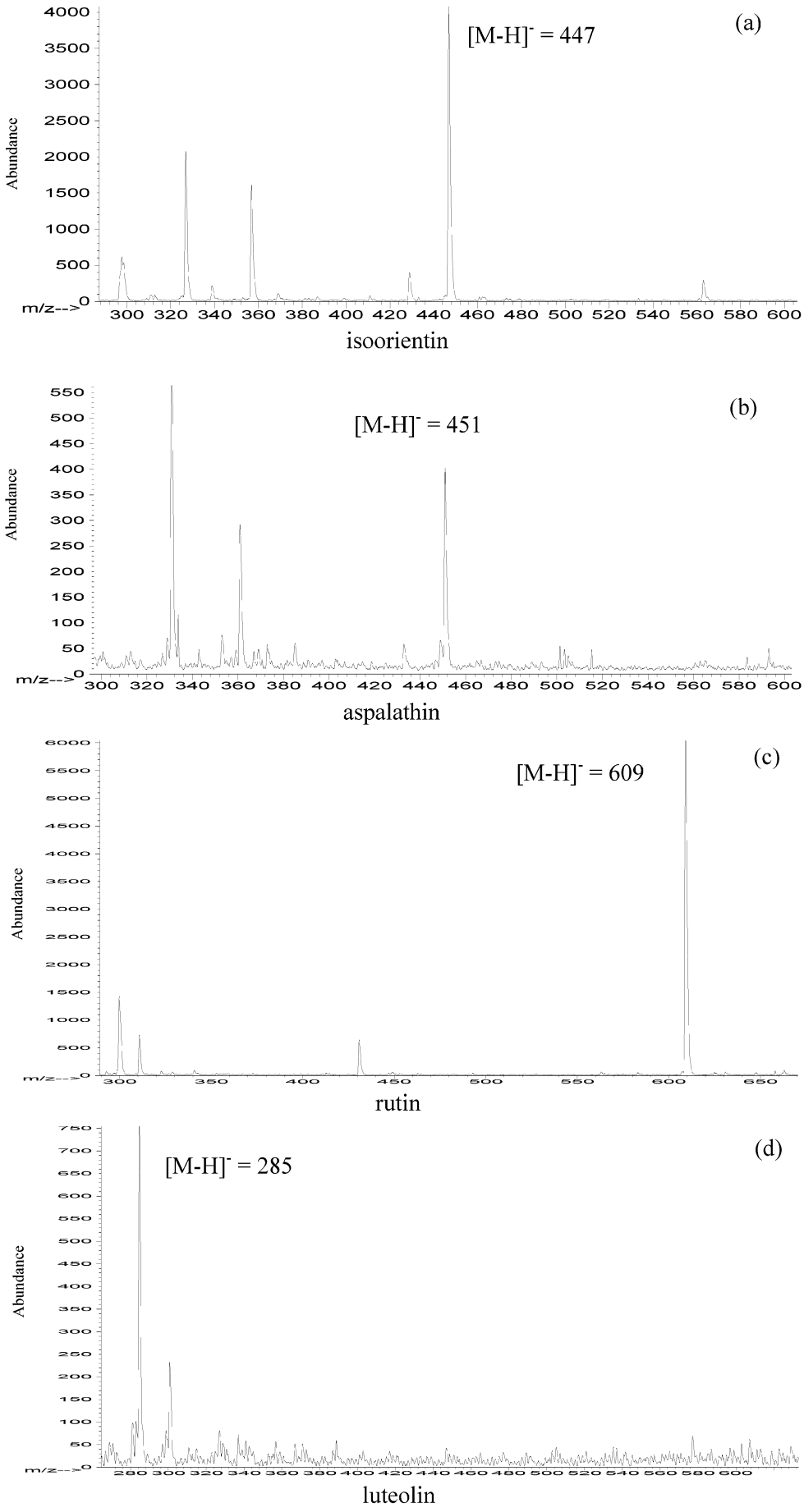

Figure 4. MS online spectra in the negative scan mode of isoorientin (a), aspalathin (b), rutin (c), and luteolin (d) in Rooibos tea infusion.

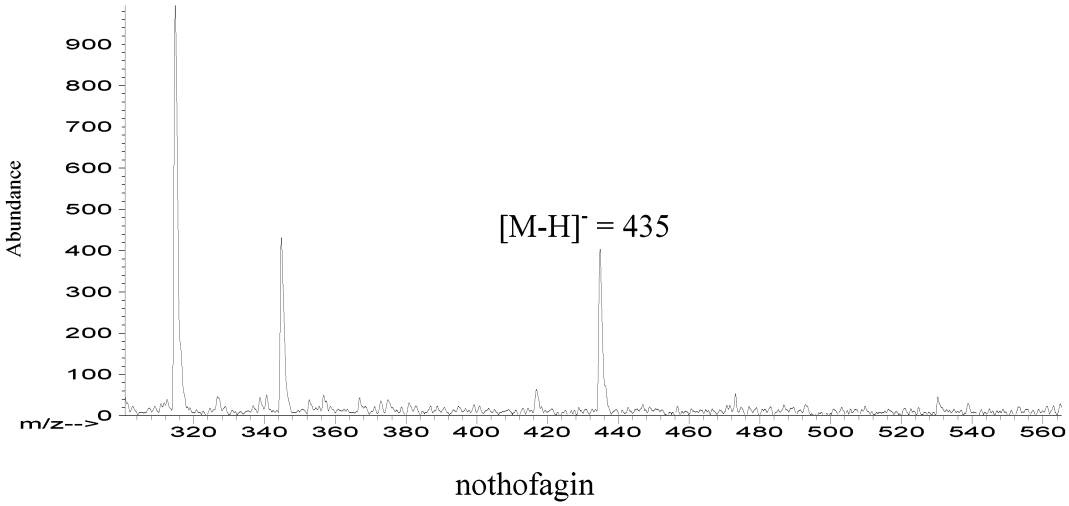

Figure 5. MS online spectra in the negative scan mode of peak c (nothofagin) in Rooibos tea.

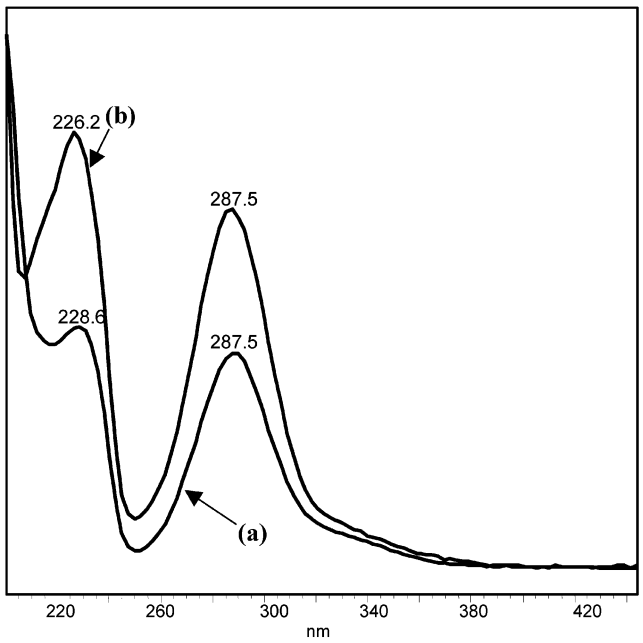

Figure 6. UV-spectra of aspalathin (a) and peak c (nothofagin) (b) in Rooibos tea.

min), dihydro-orientin (RT 10.7 min), and nothofagin (RT 15.5 min), respectively. An example is given in **Figure 5**, which shows the MS-online spectrum of peak C (corresponding to nothofagin). In addition to MS spectra, UV spectra also confirmed our suggestions concerning the identity of the peaks A, B, and C. As shown in **Figure 6**, the UV spectra of peak C (nothofagin) is comparable to that of aspalathin (structurally similar to this compound). UV spectra of the other two peaks (A and B) resulted in good accordance with those of isoorientin and orientin.

As it can be seen in **Figure 2a**, the peak with the same retention time (16.4 min) of isoquercitrin standard and m/z = 463 is not well resolved. This is due to the co-presence of isoquercitrin (quercetin-3-*O*-glucoside) and hyperoside (quercetin-3-*O*-galactoside), which cannot be differentiated by HPLC. Thus, the peak was collected five times. After concentration under vacuum, the residue was dissolved with 250 μL of methanol and the same volume of distilled water was added. This solution was analyzed by micellar electrokinetic capillary electrophoresis, which allows the separation of isoquercitrin and hyperoside (*14*). In fact, by this approach isoquercitrin and hyperoside were resolved, as confirmed by the migration times of corresponding standards. For quantitative purposes, the peak was quantified as isoquercitrin, because the absorption is essentially based on the aglycon moiety.

To determine the content of the flavonoids identified, calibration curves were prepared in the range from 0.1 to 20 μg mL^{-1}. Absorbance at λ = 255 nm (except for aspalathin at λ = 287 nm) increased linearly for all standards over the indicated concentration range (**Table 3**). The low limit of detection was 50 ng/mL.

Quantitative analyses of flavonoids present in both extracts were performed in duplicate and the results are shown in **Table 4**. As expected, methanol extraction provided levels of single and total flavonoids only slightly higher than those detected in the aqueous extract. This is relevant for in vivo studies, where aqueous infusions have to be consumed. The main compounds present in the extracts were aspalathin, rutin, and orientin, followed by isoorientin and isoquercitrin. The levels of the dihydrochalcones aspalathin and nothofagin are in good agreement with the data available in the literature (*5*). Inter- and intraday values were highly reproducible for all flavonoid compounds, as shown in **Table 5**.

In conclusion, the described method allows a sensitive and reproducible quantitation of the main flavonoids from Rooibos tea, and it may be applied to pharmacokinetic studies on this herb.

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