Characterization of the Phenolic Composition of Rooibos (*Aspalathus linearis*) RedEspresso of the system Delta Q”

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ABSTRACT

Rooibos, or *Aspalathus linearis*, is a shrub native of a mountainous region in South Africa, which has been used for more than three centuries as an herbal tea, by the local natives. This use popularity lies in the fact that its phenolic composition grants it a wide variety of health benefits.

RedEspresso, the product commercialized by Delta Cafes, consists of an espresso made from rooibos plant. Through this espresso extraction, RedEspresso results in a full-bodied drink richer and better tasting than a simple cup of rooibos tea.

This work involves analyzing and quantifying the major phenolic compounds present in a capsule RedEspresso and compare it with Rooibos infusions. The main focus is to prove that the amount of aspalathin present in a single rooibos espresso is higher than what it is present in the infusion. Given that the preparation of the espresso occurs rapidly and it’s not exposed to the environment, the aspalathin flavonoid should not be so oxidized. This way, a higher antioxidant capacity should be found in the Delta Q RedEspresso.

The separation, characterization and quantification of the major compounds, both in espresso and in infusions, was obtained through reverse phase high efficiency liquid chromatography with diode array detector, coupled to tandem mass spectrometry with electrospray ionization (RP-HPLC-DAD-ESI-MS/MS).

The validation of the analytical method was only based on the influence of the samples repeatability, the remaining calculations were limited since the extract is encapsulated.

The obtained results could not allow a precise conclusion about the capsule antioxidant capacity when compared with a regular infusion. The standards and/or the extracts deterioration at normal temperature, as well as instrumental parameters, factors that could affect the ionic chromatogram reproducibility, might be the cause of such results.

INTRODUCTION

Rooibos is a leguminous shrub native of the region of Cedarberg Mountains in South Africa, that has been used for more than 300 years by the indigenous as an herbal medicine tea. This medium bush, not exceeding 1,5 m in height is characterized by its green, glossy leaves, in the form of small needles from 1 to 4 cm. The leaves and thin stem of the plant are used for the production of Rooibos tea. They are cut into 3-4 mm lengths, fermented by enzymes from the leaves and dried in the sun (Bramati et al., 2002) . During the fermentation, the leaves acquire a typical red-brownish tonality and the aroma of the moist tea changes from resinous, hay-like and grassy to sweet, apple-like or honey-caramel. (McKay L. & Blumberg B., 2006)

Rooibos tea is a drink rich in volatile, polyphenols and mineral components. Contrary to *Camellia sinensis* tea, rooibos is caffeine-free and has a low tannin content. Thanks to these characteristics rooibos tea rapidly gained popularity as a healthy beverage.

Rooibos has a unique phenolic composition as it is the only known natural source of aspalathin. In addition, it is a source of flavone *C*-glycosides, not normally found in the daily diet. Being a natural product, free of any additives, preservatives and dyes, without caffeine content and containing natural antioxidants, which combat weakening of the body, several studies suggest that Rooibos contributes to numerous health benefits.

Delta Q system is an exclusive and patented coffee, and now tea, capsule system. Packaged in a protective atmosphere, the content in each capsule assures a standard of quality and sensory characteristics that are constant in each dose from the first to the last day of its validity. ("DELTA Q," n.d.)

In 2009, Delta Cafés bet again on innovation and launches an encapsulated rooibos, the first express made with red Rooibos in the world. An innovative drink, 100% natural, which proved to be a success, especially among the female audience, given the properties it presents. The Delta Q product Red Espresso is a full-flavored espresso made from Rooibos.

In the present work, an optimized technique of HPLC-ESI-MS/MS was applied to separate, characterize and quantify the main phenolic compounds present in the espresso extracts of Rooibos, prepared through the Delta Q system, and in the respective regular infusion.

EXPERIMENTAL SECTION

Chemicals

Methanol and acetonitrile LC-MS grade from Fisher Scientific (Loughborough, Leicestershire, UK), formic acid from Agros Organics (Geel, Belgium) and deionized water (Millipore Simplicity® Simpak 2, R = 18.2 MΩ.cm, USA) were used in all extractions and mobile-phase separations. The standards rutin, orientin and aspalatathin were purchased from Sigma-Aldrich (Steinheim, Germany). The aspalathin standard was stored at -20 ° C, while the other two standards were kept at 5 ° C.

Rooibos Infusions

Commercial packages containing capsules Red Espresso and samples of the fermented Rooibos used in encapsulation were provided by Delta Cafés.

The Red Espresso capsule was obtained by extraction with the Qool Delta's manual Q machine. Before each extraction, the machine was purged with Millipore water, to ensure that there was no tea waste in the system. Each capsule contains 4,5 g of Rooibos which was extracted with 50 ml of water during about 30 seconds. It was prepared two working solutions with a concentration of 4500 ppm, one immediately after the extraction, and other 5 hours after the extraction.

The rooibos infusions were prepared from 1,5 g of fermented rooibos tea and placed in 150 mL of millipore water at 100 ° C for about 5 min. Working solutions were prepared taken aliquots of the infusion at t = 0 min, 5 min and after 5 hours.

A solution of rutin was prepared by weighing 1 mg of rutin and diluting it in 5 mL of methanol, resulting in a concentration of $3{,}28\times10^{-4}$ M. From this solution, calibration solutions were prepared, in a range between 0,12 and 0,84 ppm.

A solution of orientin was prepared by weighing 1 mg orientin and diluting it in 5 ml of methanol, resulting in a concentration of $4{,}5\times10^{-4}$ M. From this solution, calibration solutions were prepared, in a range between 0,26 and 0,09 ppm.

The solution of standard aspalathin was prepared by weighing 0.25 mg of aspalathin and diluting it in 5 ml of methanol, resulting in a concentration of $1{,}1\times10^{-4}$ M, that was stored at -20 ° C, as recommended by the manufacturer.

Apparatus

The rooibos extracts were first analyzed in a HPLC-MS-DAD system, composed by a ProStar 410 autosampler, two chromatographic LC-210 pump, a ProStar335 diode array detector (Varian, Inc.) coupled to a mass spectrometer Ion Trap LCQ-MS 500 Fleet, equipped with an ESI ion source (Thermo Scientific). A sample (20 µL) was injected into the column through a Rheodyne injector with a 100 µL loop, in the pickup injection mode. HPLC separation was conducted on a Kinetix 100 A C18 column (150mm x 4,60mm, 5µm Phenomenex) using a mobile phase of 0,1% (v/v) formic acid (A) and acetonitrile (B), and a gradient elution programme of 0 - 20 min, linear gradient from 5% to 20% B; 20-25 min, linear gradient from 20% to 40% B; 25-30min, linear gradient from 40% to 5% B; and 30-35 min isocratic gradient of 5% B. A flow rate of 0.800 mLmin-1 was used, and the LC effluent was introduced into the ESI source in a post-column splitting ratio of 3:1: The UV-Vis spectra were recorded between 200 and 700 nm, and the chromatographic profiles were registered at the c.d.o. of 280 and 350 nm. The analysis of DAD chromatograms was carried out using Varian MS 6.9.3 Control Workstation software.

LC-MS assays were performed with a HPLC Dionex Ultimate 3000, comprising a binary pump HPG3200, an autosampler WPS300 and a column oven TCC3000 coupled in-line with a mass spectrometer LCQ Fleet ion Trap, with an ESI ion source (Thermo Scientific). A sample (10 µL) was injected into the column through a Rheodyne injector with a 25 µL loop. The separations were performed at a controlled temperature of 30 ° C, at a flowing rate of 0,350 $mLmin^{-1}$, through a Kinetix 100 A C18 column (150mm x 4,60mm, 5µm, Phenomenex), using the following elution gradient: 0- 20 min linear gradient from 5% to 15% B, 20-25 min linear from 15% to 50% B, 25-30 min linear from 50 to 100% B, 30-35 min isocratic 100% B and 35-40 min 100% at 5% B. The column was re-equilibrated for 10 minutes. The mass spectrometer was operated in the ESI negative ion modes, with the following optimized parameters: ion spray voltage, -4.8 kV; capillary voltage, -18 V; tube lens offset, 58 V; sheath gas (N_2), 80 arbitrary units; auxiliary gas (N_2), 20 arbitrary units; capillary temperature, 300 ºC. Spectra typically correspond to the average of 20–35 scans and were recorded in the range between 100–1500 Da. Tandem mass spectra (collision induced dissociation experiments) were obtained with an isolation window of 2 Da, a 20-30% relative collision energy and with an activation energy of 30 msec. Data acquisition and processing were performed using the Xcalibur software.

RESULTS

RP-HPLC-DAD-ESI-MS/MS was used to separate and identify the phenolic compounds on Rooibos extracts. The quantification was only made to rutin, orientin and aspalathin once they are the main flavonoids present on *Aspalathus linearis.* (Bramati e col,., 2002) (McKay L. & Blumberg B., 2006)

The experimental parameters for the MS and MS / MS tests were optimized with rutin, orientin and aspalathin standards. The MS/MS spectrum allowed to identify and characterize the structure of some compounds based on the characteristic fragmentation patterns. Experiments were carried out in positive and negative ESI mode, but the best results were obtained in the negative ESI mode, once they appeared to be more selective and sensitive for each family of phenolic compounds.

Phenolic compounds absorb in the ultraviolet range (UV). However, there is no single wavelength that detects all classes of compounds. The typical UV-VIS spectra of flavonoids include two absorption bands, one band I (310-385 nm) representing the absorption of the B ring and band II (250-285 nm) that corresponds to the A ring absorption.

All information obtained for the main compounds identified in the both extracts of Red Expresss and Rooibosinfusion, including the retention times, absorption spectra and mass spectra data are summarized in Table 1 (appendix).

Standard rutin

Rutin is a di-*O*-glycoside that showed a peak at *m/z* 609 on the mass spectrum, corresponding to the deprotonated molecule $[M-H]^-$. The *m/z* 609 ion was isolated on the ion trap and dissociated by collision induced with helium. The MS^2 spectrum (Figure 1) showed a small peak at *m/z* 463 formed by the loss of a rhamnose residue (-146 Da). The loss of rutinose (rhamnose + glycose residues, corresponding to 308 Da) leaded to the base peak at *m/z* 301 attributted to the quercetin agycone (**Y_0^-**).. The spectrum also presents a peak at *m/z* 300 formed by loss of the radical $H^\bullet$ from the aglycone ion Y_0^-, confirming the presence of a di-*O*-glycoside. (Vukics Viktoria; Guttman, 2010) The dissociation of the ion *m/z* 301 by cleavage of the C-ring leaded to the formation of the fragment *m/z* 179 ($^{1,2}\mathbf{A}_0^-$), which is diagnostic of the flavonols class. A proposed fragmentation pattern for the deprotonated molecule of rutin is shown in Figure 3. The peak of rutin appears on the ionic chromatogram at a retention time of 33,1 min, and its UV spectrum shows 2 bands at 256 and 353 nm, typical of the C-*O*-flavonols. (Rijke e col., 2006).

Standard Orientin

Orientin is a C-8-glycoside of luteolin, and have an isomer, the iso-orientin, when the glucose is attached to the C-6 carbon. When analyzed by (-)ESI-MS originated a peak at *m/z* 447. The MS/MS spectrum of the precursor ion *m/z* 447 ion showed losses of 120 and 90 Da, typical of the *C*-glycosides and confirming the presence of a hexose. Less intense peaks at *m/z* 411 and 369 are due to losses of H_2O molecules or conjugated losses of H_2O and fragmentations in the glucose unit. The very low abundant ion at *m/z* 285 formed by the loss of 162 Da (glucose residue) leads to the deprotonated aglycone of luteolin, the ion Y_0^- (*m/z* 285). Figure 2 shows the proposed fragmentation for the deprotonated molecule orientin m/z 447. LC-MS/MS analysis of standard orientin gave a peak at Rt = 28,3 min and the UV-Vis spectrum shows two bands at λmax 269 and 348 nm, confirming the presence of a *C*-glycoside and a flavones, respectively (Pinheiro & Justino, 2012)

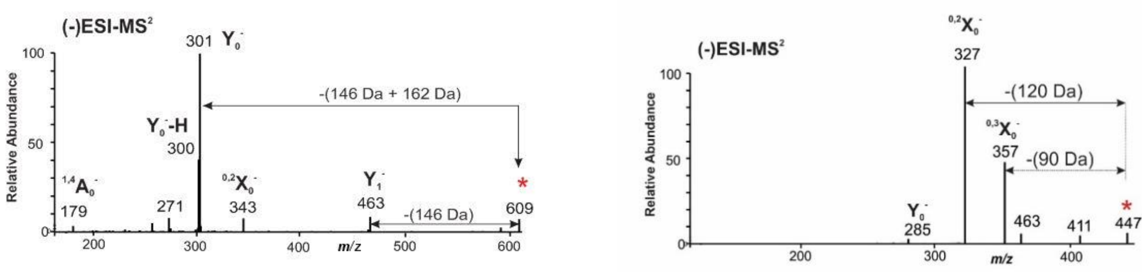


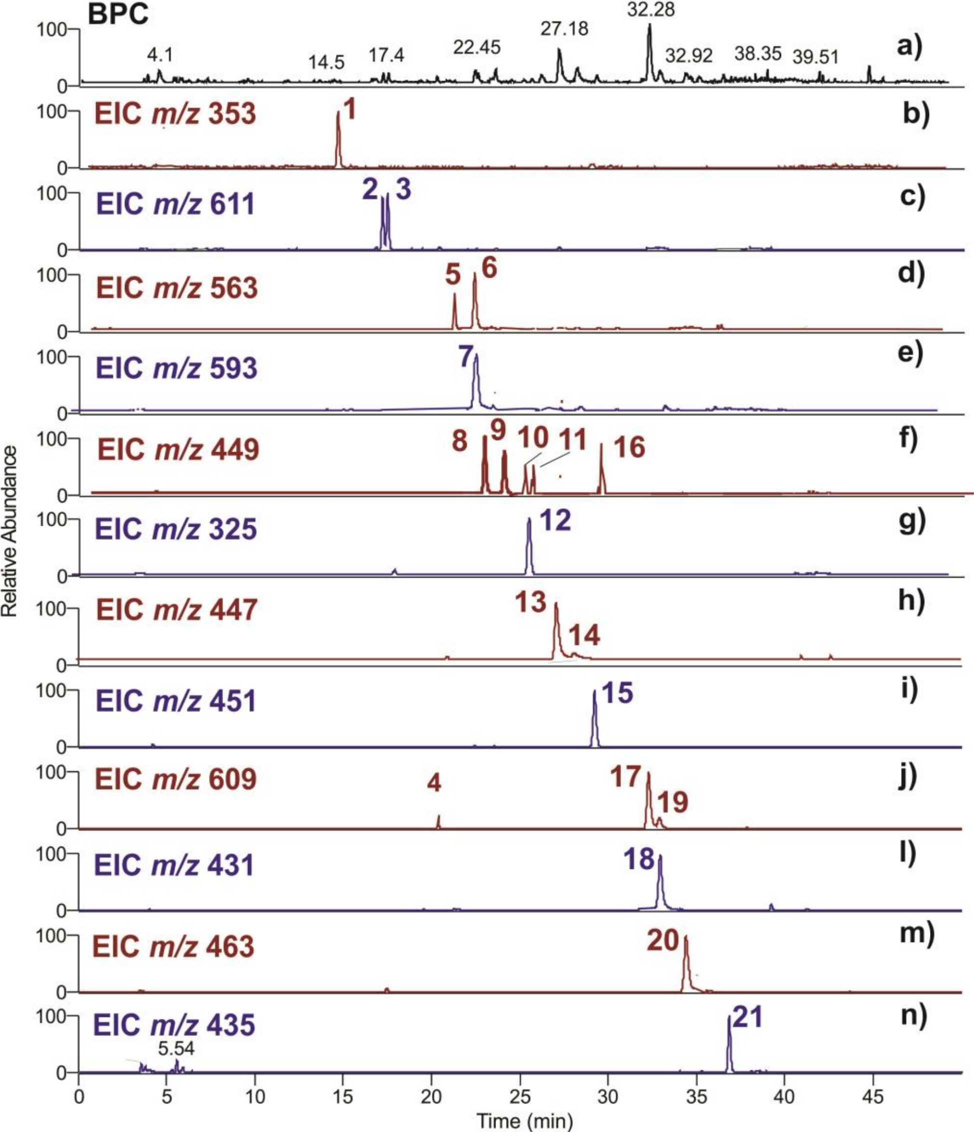
Figure 1- ESI-MS^2 Spectrum of the deprotonated molecules rutin m / z 609 and orientin m/z 447

Figure 2 – Proposed fragmentation for rutin (left scheme)and orientin (right scheme)..

Standard aspalathin

As orientin, aspalathin is a *C*-glycoside classified as a hydrochalcone. The mass spectrum in the negative ESI mode showed a peak at *m/z* 451, attributed to the deprotonated molecule of asphalathin.. This precursor ion when subjected to CID, shows a fragmentation pattern identical to that of orientin, typical to the *C*-glycosides class. In our HPLC conditions, aspalathin shows a Rt = 29,2 min. Its UV-Vis spectrum showed one band at 286 nm, typical of the chalcones. It is important to refer that at room temperature (21ºC) this compound is not stable, occurring degradation with formation of the C-glucopyranoside derivatives of luteolin.

Figure 3 shows the total ion chromatogram obtained in the negative ESI mode and the extracted ion chromatograms for the main phenolic compounds identified in both the Red express and infusion extracts. As mentioned before, the compounds were identified based on their retention times, absorbance spectra, MS spectra and MS/MS fragmentation patterns, by comparison with published data and with the available standards. (Table 1 appendix)



BPC
100
0
4.1
14.5
17.4
22.45
27.18
32.28
32.92
38.35
39.51
a)
EIC m/z 353
1
b)
EIC m/z 611
2
3
c)
EIC m/z 563
5
6
d)
EIC m/z 593
7
e)
Relative Abundance
EIC m/z 449
8
9
10
11
16
f)
EIC m/z 325
12
g)
EIC m/z 447
13
14
h)
EIC m/z 451
15
i)
EIC m/z 609
4
17
19
j)
EIC m/z 431
18
l)
EIC m/z 463
20
m)
EIC m/z 435
5.54
21
n)
0
5
10
15
20
25
30
35
40
45
Time (min)

Figure 3 - Analysis HPLC-MS/MS of a RedEspresso extract. a) Base peak chromatogram in the negative ESI mode. Extracted ion chromatograms for precursors b) m/z 353; c) m/z 611; d) m/z 593; e) m/z 593; f) m/ z 449; g) m/z 325; h) m/z 447; i) m/ z 451; j) m/z 609; l) m/z 431; m) m/z 463 and n) m/z 435

To determine the content of the main flavonoids identified, calibration curves of rutin and orientin were prepared. It was found that asphalathin was not stable when dissolved in water or methanol, unabling the validation of its calibration curve. The standards of the C-glycosides of eriodictyol were not available on the market.

Quantitative analysis was performed in both extracts, from tea capsule, directly extracted from the machine, and in the infusions. Samples were collected at different times, 0 min, 5 min to 5 h, in order to examine its influence on the concentration of the compounds.

The methodology developed was based on the external standard method. Solutions whose concentrations vary in the case of rutin, from 0.12 to 0.84 ppm, and in the case of orientin between 0.26 and 0.09 ppm were analyzed by LC-MS/MS, under the same conditions as the samples to be analyzed.

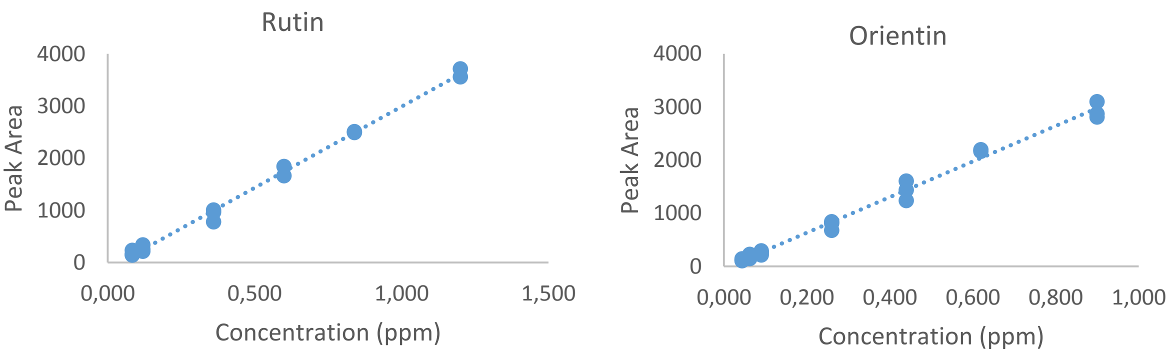


Figure 4 – Calibration curves obtained for rutin and orientin.

Table 2 - Obtained parameters for the calibration curves.

Rutin Calibration Curve	R^2	LQ (ppm)	LD (ppm)
$y = 3117,3\,x - 124,89$	0,9956	0,276	0,091
Orientin Calibration Curve	**R^2**	**LQ (ppm)**	**LD (ppm)**
$y = 3347,1\,x - 36,026$	0,9905	0,312	0,103

To validate the analytical methodology only the influence of repeatability was studied. Since the study involves the analysis of a sample that is enclosed in a capsule, it was not possible to calculate the recovery, which gives a measure of the efficacy of the analytical method. The repeatability of the method was evaluated by calculating the accuracy of separate day and intraday assays. The intraday precision was evaluated with different values for standard

concentration, by analyzing three replicates for each concentration held on the same day. In the case of orientin concentrations were 0,06, 0,44 and 0,90 ppm, and for rutin 0,08, 0,60 and 1,20 ppm. The standard deviation was calculated (SD), coefficient of variation (CV) as well as the standard deviation weighed and repeatability for each compound. (Tables 5 and 6 in appendix)

Since aspalathin, nothofagin and eriodictyol-glicosides are structurally similar to orientin, i.e. they are C-glycosides, it was expected that they have an identical behavior when analysed under the some ESI conditions. Assuming that all these flavonoids display similar ESI responses, it was used the calibration curve of orientin to estimate the concentration of aspalathin, nothofagin and erioctyol-glicosides in both tea an infusion extracts. Table 3 and 4 summarizes the results obtained for all the compounds quantified.

Table 3 – Obtained concentrations of rutin and orientin (and its isomers) in the capsule and in the infusions.

Samples	Rutin	Rutin Isomer	Iso-Orientin	Orientin
	Rt=32,5	Rt=33,2	Rt=27,4	Rt=28,4
Capsule	**C (mg/4,5g rooibos)**			
t=0min	0,137	0,038	0,105	0,098
t=5h	0,136	0,035	0,130	0,099
Infusions	**C (mg/1,5g rooibos)**			
t=0min	0,195	0,055	0,197	0,156
t=5min	0,184	0,058	0,223	0,201
t=5h	0,283	0,085	0,216	0,243

Table 4 - Obtained concentrations of aspalathin, nothofagin and eriodictiol in the capsule and in the infusions.

Samples	Aspalathin	Nothofagin	Eriodicityol Derivatives				
	Rt=29,5	Rt=36,9	Rt=22,7	Rt=23,7	Rt=25,3	Rt=25,9	Rt=29,6
Capsule	**C (mg/4,5g rooibos)**						
t=0min	0,063	0,008	0,032	0,028	0,013	0,005	0,010
t=5h	0,031	0,006	0,024	0,022	0,013	0,004	0,008
Infusions	**C (mg/1,5g rooibos)**						
t=0min	0,054	0,010	0,033	0,024	0,019	0,007	0,012
t=5min	0,044	0,012	0,036	0,033	0,023	0,008	0,011
t=5h	0,060	0,011	0,043	0,025	0,017	0,009	0,014

CONCLUSIONS

The HPLC-ESI-MS/MS methodology used to identify and quantify the phenolic compounds present in both Red Espresso and infusion extracts meets the aforementioned literature, proved to be suitable for the separation and characterization of the compounds.

The data clearly show that in both, the capsule and the infusion extracts, rutin and iso-orientin comprise the majority of the flavonoids. In general, the infusions were shown to have a higher concentration of flavonoids. Considering the concentration of aspalathin, the data are inconclusive, as it appears to suggest that both capsule and infusion when analyzed at once after the addition of tea, have identical concentrations of this compound.

Keeping the tea bag in the infusion and exposed to air for several hours seems to have little influence on the variation of the majority of the compounds, since only for rutin and orientin were found a clear increase in concentration at higher times.

When the espresso was left exposed to air for around 5 hours, it was observed a decrease in the concentration of aspalathin and an increase in the concentration of iso-orientin. This result may indicate that when the infusion is exposed to air for a long time, degradation of aspalathin occurs.

As the analytical method was not validated, since there are a lack of reproducibility in the signal response of the ionic chromatograms, the results are merely indicative, and do not allow an accurate quantitation of the phenolic constituents present in the espresso and infusion extracts. Consequently, the main objective of this work, to infer if the antioxidant capacity of the capsule is higher than that of the infusion, was not achieved.

For future work a study of the stability of the aspalathin standard solutions should be made, in order to validate an analytical method that enables accurate quantification of this compound in the extracts of the capsule and infusion. In addition a re-optimization of the experimental conditions is necessary, since data spread appears to result from the lack of reproducibility of the mass spectrometer response. The variation of the ion signal can be related to the fact that the flow rate of the mobile phase used in the analytical method (0,350 $mLmin^{-1}$) is too high, inhibiting the optimal ESI nebulization conditions of the samples.

References

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P., Pietta, P. (**2002**). Quantitative Characterization of Flavonoid Compounds in Rooibos Tea (Aspalathus linearis) by LC − UV / DAD. *Journal of Agricultural and Food Chemistry*, 50, 5513–5519.

Delta Cafés. (**2015**). Retrieved April 5, 2015, from http://www.delta-cafes.pt/

McKay L. D., Blumberg, B. J. (**2006**). Review of the bioactivity of South African Herbal Teas: Rooibos (Aspalathus linearis) and Honeybusg (Cyclopia intermedia). *Phytoterapy Research*, 21(21), 2695–2700

Pinheiro, P., Justino, G. (**2012**). Structural analysis of flavonoids and related compounds—a review of spectroscopic applications. *Phytochemicals – A Global Perspective of Their Role in Nutrition and Health*, 33–56.

Rijke, E., Out, P., Niessen, W. M. A., Ariese, F. Gooijer, C., Brinkman, U,.A. (**2006**). Analytical separation and detection methods for flavonoids. *J. Chromatogr. A.*, 1112, 31–63.

Vukics, V.; Guttman, A. (**2010**). Structural Characterization of flavonoid glycosides by multi-stage mass spectrometry. *Mass Spectrometry Reviews*, 29, 1–16.

Appendix

Table 1 - Identification by HPLC-ESI-MS/ MS of the phenolic compounds present in both extracts of rooibos RedEspresso and infusion.

Peak	Rt *(min)*	λmax *(nm)*	Structure	[M-H]⁻ *(m/z)*	MS^2 (*m/z*, %, Ion attribution*)*	Proposed Compound
1	14.5	293		353	MS^2 [353] :191(90) [M-H-152]⁻, 179 (100) [M-H-146]⁻, 123 (80) [M-H-202]⁻	caffeoylquinic acid
2	17.4	277		611	MS^2 [611]: 593 (10) [M-H-18]⁻, 521 (20) [M-H-90]⁻, 491 (100) [M-H-120]⁻, 401 (60) [M-H-(90+120)]⁻ 371 (80) [M-H-2x 120]⁻	Safflomin A
3	17.7	277		611	MS^2 [611]: 521 (20) [M-H-90]⁻, 491 (100) [M-H-120]⁻, 401 (60) [M-H-(90+120)]⁻, 371 (80) [M-H-2x120]⁻	Safflomin A isomer
4	20.5	273, 346	R_1=H; R_2=OH;R_3=H; R_4=glucose	609	MS^2 [609]: 519 (30) [M-H-90]⁻, 489 (100) [M-H-120]⁻, 447 (20) [M-H-162]⁻, 399 (20) [M-H-(90+120)]⁻ 369 (20) [M-H-(2x 120)]⁻	Luteolin-*C*-di-glycoside
5	21.5	278, 330	R_5=H; R_6=H;R_7=arabinose	563	MS^2 [563]: 545 (10) [M-H-18]⁻, 503 (60) [M-H-60]⁻, 473 (100) [M-H-90]⁻, 443 (40) [M-H-120]⁻, 383 (80) [M-H-(60+120)]⁻, 353 (70) [M-H-(90+120)]⁻	Isoschaftoside

6	22.9	278, 330				563	MS^2 [563]: 503 (30) [M-H-60]$^-$, 473 (100) [M-H-90]$^-$, 443 (90) [M-H-120]$^-$,383 (70) [M-H-(60+120)]$^-$, 353 (90) [M-H- (90+120)]$^-$	Apigenin (6-*C*-β-D-glucopyranosyl) 8-*C*-α-L-arabinoside (Schaftoside)
7	22.9	273, 332				593	MS^2 [593]: 503 (20) [M-H-90]$^-$, 473 (100) [M-H-120]$^-$, 383 (50) [M-H-(90+120)]$^-$, 353 (60) [M-H-(2x120)]$^-$	Vicenin II
8	22.7	280				449	MS^2 [449]: 413 (2) [M-H-18]$^-$,359 (10) [M-H-90]$^-$, 329 (100) [M-H-120]$^-$	S-eriodictyol-6-*C*-β-D-glucopyranoside
9	23.7	280				449	MS^2 [449]: 413 (3) [M-H-18]$^-$,359 (5) [M-H-90]$^-$, 329 (100) [M-H-120]$^-$	R-eriodictyol-6-*C*-β-D-glucopyranoside
10	25.3	280				449	MS^2 [449]: 359 (8) [M-H-90]$^-$, 329 (100) [M-H-120]$^-$	S-eriodictyol-8-*C*-β-D-glucopyranoside
11	26.4	280				449	MS^2 [449]: 359 (5) [M-H-90]$^-$, 329 (100) [M-H-120]$^-$	R-eriodictyol-8-*C*-β-D-glucopyranoside
12	26.2	280				325	MS^2 [325]: 163(90) [M-H-162]$^-$, 119 (100) [M-H-162-44]$^-$, 102 (25) [M-H-162-(44+18)]$^-$	Coumarin glucoside

13	27.4	270, 349		447	MS^2 [447]: 429 (20) [M-H-18]$^-$, 357 (80) [M-H-90]$^-$, 327 (100) [M-H-120)]$^-$	Iso-Orientin
14	27.4	270, 349		447	MS^2 [447]: 357 (40) [M-H-90]$^-$, 327 (100) [M-H-120)]$^-$	Orientin
15	29.5	287		451	MS^2 [451]: 433 (4) [M-H-18]$^-$, 361 (15) [M-H-90]$^-$, 331 (100) [M-H-120)]$^-$	Aspalathin
16	29.5	287		449	MS^2 [449]: 359 (20) [M-H-90]$^-$, 329 (100) [M-H-120)]$^-$, 285 (20) [M-H-120-44)]$^-$	Aspalathinin
17	32.5	256, 352		609	MS^2 [609]: 463 (2) [M-H-162]$^-$ **(Y_1^-)**, 343 (20) [M-H-162-120]$^-$ **($^{0,2}X_0^-$)**, 301 (100) [M-H-308]$^-$**(Y_0^-)**, 300 (80) **(Y_0-H•)**	Rutin
18	33.2	270, 331		431	MS^2 [431]: 413 (7) [M-H-18]$^-$, 341 (20) [M-H-90]$^-$, 311 (100) [M-H-120)]$^-$	Iso-vitexin
19	33.2	-		609	MS^2 [609]: 343 (5) [M-H-162-120]$^-$ **($^{0,2}X_0^-$)**, 301 (100) [M-H-308]$^-$ **(Y_0^-)**, 300 (40) **(Y_0-H•)**	Quercetin-di-*O*-glucoside

20	34.6	254,346			463	MS^2 [463]$^-$ 301 (100) [M-H-308]$^-$ **(Y_0^-)**, 300 (50) **(Y_0-H$^\bullet$)**, 179 (7) ^{1,2}**A**$^-$, 151 (5) ^{1,2}**A**$^-$-CO	Quercetin-3-*O*-glucoside
21	36.9	287			435	MS^2 [435]: 345 (30) [M-H-90]$^-$, 315 (100) [M-H-120)]$^-$	Nothofagin

Rt- retention time ; [M-H]$^-$ deprotonated molecule, *m/z* - mass/charge; ESI-MS/MS – tandem mass spectrometry with electrospray ionization

Table 5 - Precision parameters obtained for orientin.

Rutin Interdays n=9					
Concentration (ppm)	**Concentration average (ppm)**	**DP**	**CV(%)**	**Pondered standard deviation**	**Repeatability**
0,08	0,10	0,0055	5,7	0,0246	0,069
0,60	0,58	0,0263	4,6		
1,20	1,21	0,0332	2,7		
Rutin Intradays					
Concentration (ppm)	**Concentration average (ppm)**	**DP**	**CV(%)**	**Pondered standard deviation**	**Repeatability**
1,200	1,127	0,0347	3,1	0,0280	0,078
0,840	0,762	0,0051	0,7		
0,600	0,522	0,0410	7,9		
0,360	0,253	0,0381	15,0		
0,120	0,046	0,0196	42,9		
0,084	0,017	0,0161	96,0		

Table 6 - Precision parameters obtained for rutin.

Orientin Interdays n=9					
Concentration (ppm)	**Concentration average (ppm)**	**DP**	**CV(%)**	**Pondered standard deviation**	**Repeatability**
0,062	0,020	0,0058	29,8	0,0435	0,122
0,440	0,335	0,0179	5,3		
0,900	0,646	0,0529	8,2		
Orientin Intradays					
Concentration (ppm)	**Concentration average (ppm)**	**DP**	**CV(%)**	**Pondered standard deviation**	**Repeatability**
0,260	0,222	0,0256	11,5	0,0305	0,085
0,440	0,417	0,0551	13,2		
0,044	0,026	0,0049	18,8		
0,620	0,642	0,0068	1,1		
0,062	0,043	0,0133	30,9		
0,900	0,864	0,0452	5,2		
0,090	0,066	0,0121	18,4		