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Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given simultaneously through diet

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1 **Title**

2 Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given
3 simultaneously through diet

4

5 **Running title**

6 Relative bioavailability of flavonoids in humans

7

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30 **Authors' contribution**
31 KSK: contribution to the study design and responsible for conducting the experiment, responsible
32 for LC-MS analysis of urine, plasma and juice, data interpretation and writing the manuscript;
33 LB: contribution to the LC-MS analysis of plasma and juice, data interpretation and manuscript
34 editing; PK: responsible for the LC-MS analysis of diet, contribution to the LC-MS analysis of
35 'juice-mix' and manuscript editing; JH: contribution to the study design and conducting the
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40

41 **ABSTRACT**

42 The bioavailability and urinary excretion of three dietary flavonoids, quercetin, hesperetin and
43 naringenin was investigated. Ten healthy men consumed a 'juice-mix' containing equal amounts
44 of the three flavonoids and urine and plasma samples were collected. Resulting mean plasma
45 AUC_{0-48h} and C_{max} values for quercetin and hesperetin were similar, whereas the AUC_{0-48h} of
46 naringenin, and thus the relative bioavailability, were higher after consumption of the same dose.
47 The study consolidates a significant lower urinary excretion of quercetin ($1.5\% \pm 1\%$) compared to
48 hesperetin ($14.2\% \pm 9.1\%$) and naringenin ($22.6\% \pm 11.5\%$) and shows that this is not due to a
49 lower bioavailability of quercetin, but rather reflects different clearance mechanisms.

50

51 **Keywords:** bioavailability, biokinetics, humans, quercetin, hesperetin, naringenin.

52

53 To compare the impact of dietary important flavonoids it is necessary to study them in the
54 same food matrix and at similar realistic doses. The present study investigate the bioavailability
55 and urinary excretion of the flavonoids, quercetin, hesperetin and naringenin in a 48h intervention
56 study with a single dose (6.3 mL/kg bw) of 'juice-mix' containing the three flavonoids.

57 Complete urine and plasma samples were obtained from 10 healthy men, 21-28 years.
58 The study was approved by the ethics committee of Copenhagen and Frederiksberg municipality
59 (J.No.(KF)01-161/01). The 'juice-mix' was provided to fasting subjects in the morning, along
60 with a standardized flavonoid-free diet (0-24 h), after which the volunteers kept a flavonoid-free
61 diet (24-48 h). Blood and urine samples were collected as previously described (Nielsen *et al.*
62 2006). Flavonoid aglycones were quantified in the 'juice-mix' (30 mg/L quercetin, 28 mg/L
63 naringenin and 32 mg/L hesperetin) and flavonoid glycosides were identified according to
64 Breinholt *et al.* (2003).

65 Flavonoids in plasma were completely hydrolysed as described in Nielsen *et al.* (2006).
66 Samples were added 25 μ L aqueous ascorbic acid (20 mg/mL) and 0.5% formic acid to pH = 4
67 and applied to Evolute ABN columns (25 mg, Mikrolab, Denmark). The eluted flavonoid
68 aglycones were evaporated to dryness and re-dissolved in 200 μ L 0.5% formic acid, 10%
69 methanol and added 250 ng 13 C-daidzein as external standard.

70 Determination of flavonoids in urine is essentially described elsewhere (Nielsen *et al.*,
71 2006), except for the inclusion of SPE (Isolute SPE 100) before injection on the LC-MS system.

72 Statistical analyses were performed using Wilcoxon matched pair tests (SPSS version
73 14.0). The relative bioavailability, AUC_{0-48h} , was calculated with the linear trapezoidal rule.

74

75

76 RESULTS AND DISCUSSION

77 This study demonstrates that when subjects consume equal amounts of quercetin and
78 hesperetin, the AUC_{0-48h} , and thus the bioavailabilities of these flavonoids, is similar. However,
79 the AUC_{0-48h} of naringenin was significantly higher than for quercetin and hesperetin (**Figure 1**).
80 Also plasma C_{max} reached for quercetin and hesperetin was comparable, whereas the level
81 achieved for naringenin was significantly higher (**Table 1**). In the study design we assumed that
82 the low and natural amounts of flavonoids in the 'juice-mix' excluded any interference with
83 respect to pharmacokinetics of the flavonoids investigated. Previous studies dealing with
84 flavanone and flavonol bioavailability report AUC-values in the same range (Hollman *et al.*,
85 1997; Erlund *et al.*, 2001; Manach *et al.*, 2003; Manach *et al.*, 2005) as observed in the current
86 study. However, the lack of blood sampling between 8-24 h in the present study may cause an
87 overestimation of the AUCs, especially for the flavanones.

88 In the present study, we observed a 9-15 fold higher urinary excretion of naringenin and
89 hesperetin compared to quercetin (**Table 1**) consolidating the apparent paradox that absorption
90 and bioavailability of the three flavonoids seems quite similar, but that urinary excretion is highly
91 different. The low urinary recovery (1.5%) of the ingested quercetin leaves a large amount of the
92 ingested dose unaccounted for compared to hesperetin and naringenin. The most likely fate of the
93 absorbed quercetin is excretion via bile (Matsukawa *et al.*, 2009) and further degradation to low
94 molecular weight phenolic acids which were not analysed in the current and most previous
95 flavonol bioavailability studies (Mullen *et al.*, 2006; Mullen *et al.*, 2008). Only small amounts of
96 the 3'-methylated form of quercetin, isorhamnetin, were present in the 'juice-mix' (1.8 mg/500
97 mL 'juice-mix') and of this only $4.1 \pm 3.3\%$ of isorhamnetin was excreted in urine after 48 h,
98 excluding extensive methylation of quercetin. Only trace amounts of tamarixetin (4'-OMe-
99 quercetin) were found in juice, plasma and urine.

100 Hydrolysis of the flavonoid glycoside-moiety is a necessary step for absorption of
101 flavonoids from the gut. In the present study apple juice was the source of quercetin and
102 qualitative LC-MS investigation of the ‘juice-mix’ showed that approximately 60% of the
103 quercetin was present as monoglycosides, 25% as aglycone and less than 15% as rutosides. It
104 has been shown that only quercetin-3-glucoside and quercetin-4'-glucoside is absorbed in
105 substantial quantities in the small intestine, while little quercetin-3-galactoside, and quercetin-3-
106 rhamnoside and quercetin-3-arabinoside is absorbed in the small intestines, but are hydrolysed
107 and absorbed in the distal part of the colon together with diglycosides such as naringin and
108 hesperidin (Arts *et al.*, 2004). More than 99% of the hesperetin and naringenin in the ‘juice-mix’
109 were diglycosides, presumably naringin and hesperidin. The nature of the glycoside moieties
110 bound to the three flavonoids was thus, mainly in a form that favours absorption from the colon.
111 The similar T_{max} for quercetin, hesperetin and naringenin in current study supports this
112 assumption. T_{max} for quercetin in the literature is between 1-9 h, depending on sugar moiety
113 composition (Manach *et al.*, 2005), and the intermediate T_{max} (3.6 ± 1.6 h) in the present study
114 correlates with the mixed content of quercetin glycosides. The average T_{max} in the literature for
115 naringenin is between 2.0–4.6 h (Manach *et al.*, 2003) and for hesperetin between 5.4–5.8 h
116 (Manach *et al.*, 2003) and thus also in the same range as observed here.

117 In conclusion, the current study confirms a lower urinary excretion of quercetin compared
118 to naringenin and hesperetin. The study furthermore shows that the significant lower urinary
119 excretion of quercetin is not due to a low bioavailability of quercetin, but rather reflects different
120 clearance mechanisms for this flavonoid.

121

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127

128 **TABLE 1**

129 Plasma and urinary biokinetic parameters for quercetin, naringenin and hesperetin after
 130 administration of 'juice-mix' (mean \pm SD, n=10).

	Mean excreted amount (μ g)	Accumulated relative urinary excretion (% of the dose)	T _{max} plasma (h)	C _{max} plasma (μ mol/L)	C _{max} /AUC _{0-48h}	AUC _{0-48h} (μ mol* h/L)	AUC _{0-48h} (μ mol* h/L) ¹
Quercetin (30 mg/L 'juice-mix')			3.6 \pm 1.6	0.15 \pm 0.13	0.09 \pm 0.02	1.77 \pm 1.63	1.77 \pm 1.63
0-3 h	31 \pm 13	0.3 \pm 0.2					
3-6 h	73 \pm 29	0.9 \pm 0.7					
6-12 h	74 \pm 11	1.2 \pm 0.9					
12-24 h	21 \pm 17	1.3 \pm 0.9					
24-48 h	28 \pm 18	1.5 \pm 1.0					
0-48 h	227 \pm 142 ^{b,c}	1.5 \pm 1.0 ^{b,c}					
Naringenin (28 mg/L 'juice-mix')			3.6 \pm 1.6	0.25 \pm 0.13 ^a	0.11 \pm 0.05	2.64 \pm 1.95	2.82 \pm 2.09 ^a
0-3 h	456 \pm 231	3.3 \pm 1.6					
3-6 h	1390 \pm 1060	13.2 \pm 8.2					
6-12 h	952 \pm 482	20.0 \pm 10.0					
12-24 h	278 \pm 282	22.0 \pm 10.9					
24-48 h	83 \pm 114	22.6 \pm 11.5					
0-48 h	3160 \pm 1612	22.6 \pm 11.5					
Hesperetin (32 mg/L 'juice-mix')			4.9 \pm 1.4	0.18 \pm 0.13 ^b	0.09 \pm 0.03	2.13 \pm 1.59	1.99 \pm 1.49 ^b
0-3 h	103 \pm 78	0.6 \pm 0.5					
3-6 h	802 \pm 595	5.7 \pm 3.6					
6-12 h	880 \pm 462	11.2 \pm 6.3					
12-24 h	436 \pm 542	13.9 \pm 8.7					
24-48 h	58 \pm 103	14.2 \pm 9.1					
0-48 h	2278 \pm 1457 ^b	14.2 \pm 9.1 ^b					

131 ¹Adjusted for ingested dose. Ingested dose is based on chemical analysis. The contents of flavonoids were calculated
 132 on the basis of internal and external standards.

133 ^aSignificantly different from quercetin (Wilcoxon, p<0.01).

134 ^bSignificantly different from naringenin (Wilcoxon, p<0.01).

135 ^cSignificantly different from hesperetin (Wilcoxon, p<0.01).

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138 **FIGURE LEGENDS**

139

140 **FIGURE 1** Plasma time versus concentration curves for quercetin (diamond), hesperetin
141 (triangle) and naringenin (square) after ingestion of 'juice-mix'. Values are mean + SEM, n=10.

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FIGURE 1

