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Variation in the Methylation of Caffeoylquinic Acids and Urinary Excretion of 3′-methoxycinnamic acid-4′-Sulfate After Apple Consumption by Volunteers

*Laura Rubió, Maria Paz Romero, Rosa Solà, Maria José Motilva, Michael N. Clifford, and Alba Macià**

Introduction: It has been reported that the phenolic metabolite 3′-methoxycinnamic acid-4′-sulfate generated from 5-*O*-caffeoylquinic acid may have potential benefits in human health. However, the variation in 3′- and 4′-methylation of 3′,4′-dihydroxycinnamic acid and its impact on the yield of this sulfate metabolite is unclear and has been poorly studied.

Methods and Results: To address this aim, the excreted 3′-methoxy and 4′-methoxy metabolites in urine samples (24-h) are determined in 14 volunteers after an acute intake of 80 g of red-fleshed apple (RFA) or white-fleshed apple (WFA). These methoxy metabolites are also determined in the same volunteers in a second acute intake after a 6-week sustained consumption of the same products.

Conclusion: Seven 3′-methoxy and seven 4′-methoxy metabolites are determined, i.e., the free cinnamic and corresponding phenylpropanoic acid, plus their sulfate, glucuronide, and glycine conjugates. In only six volunteers, five females and one male, is 4′-methylation preferred over 3′-methylation, but it is observed that an individual's 3′- : 4′-methylation ratio can change over time, and that the yield of 3′-methoxycinnamic acid-4′-sulfate is extremely variable, ranging from undetectable to 71% of the total C_6-C_3 metabolites excreted, and any benefit accruing from this metabolite will not necessarily be available to all consumers.

1. Introduction

A recent review of the human metabolism of acyl-quinic acids (caffeoylquinic acids) drew attention to the substantial person-to-person variation with reference to the profile of metabolites excreted in urine 24 h after consumption of a test beverage such as coffee or maté, and recommended that more data on, and a better understanding of this variation was required.[1] The crucial importance of such variation on the possible health promoting properties of foods and beverages has been discussed by Kerimi et al.[2] They drew attention to how variation in the competence of the gut microbiota to hydrogenate 3′,4′-dihydroxycinnamic acid (caffeic acid) and 3′-methoxy-4′-hydroxycinnamic acid (ferulic acid), coupled with variation in the extent of endogenous β-oxidation of these C_6–C_3 metabolites and their Phase II conjugation (glucuronide, sulfate, or glycine), would have a marked impact on the yield

L. Rubió, M. P. Romero, A. Macià
Antioxidants Research Group
Food Technology Department
Agrotecnio AGROTECNIO-CERCA Center
University of Lleida
Av/ Alcalde Rovira Roure 191, Lleida 25198, Spain
E-mail: alba.macia@udl.cat

R. Solà
Facultat de Medicina i Ciències de la Salut
Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut)
Universitat Rovira i Virgili
C/ Sant Llorenç 21, Reus 43201, Spain

M. J. Motilva
Instituto de Ciencias de la Vid y del Vino (ICVV) (Consejo Superior de Investigaciones Científicas-CSIC
Gobierno de La Rioja
Finca "La Grajera,"
Universidad de La Rioja
Carretera de Burgos km 6, 26007, Logroño, La Rioja, Spain

M. N. Clifford
School of Bioscience and Medicine
Faculty of Health and Medical Sciences
University of Surrey
Guildford, UK

M. N. Clifford
Department of Nutrition
Dietetics, and Food
School of Clinical Sciences at Monash Health
Faculty of Medicine Nursing and Health Sciences
Monash University
Notting Hill, Victoria, Australia

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202100471

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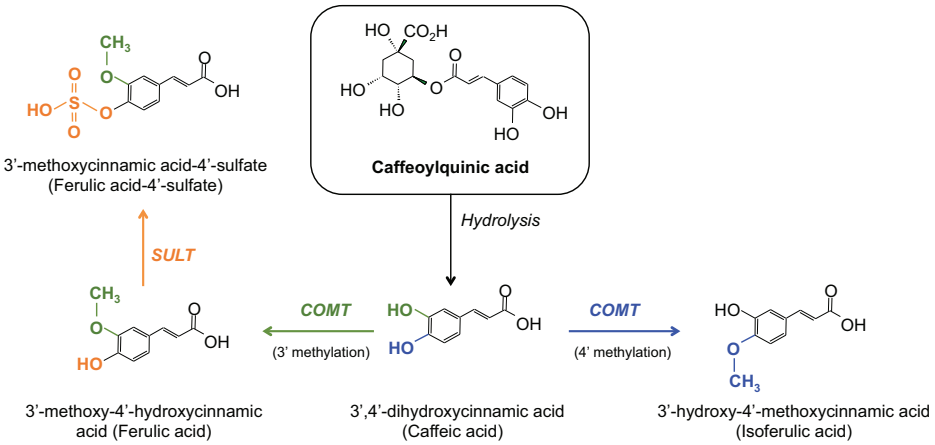

Figure 1. Schematic representation of the metabolic pathways of 3′-4′-dihydroxycinnamic acid conjugates and generate 3′-methoxycinnamic acid-4′-sulfate

of 3′-methoxycinnamic acid-4′-sulfate (ferulic acid-4′-sulfate).[2] This metabolite is of particular interest because using isolated mouse saphenous and femoral arteries and aortae in vitro it has been shown to produce vasorelaxation.[3,4] In clinical studies, 5-*O*-caffeoylquinic acid, a known dietary precursor of this metabolite (3′-methoxycinnamic acid-4′-sulfate), has been shown to reduce diastolic and systolic blood pressure,[5] and beverages containing acyl-quinic acids (such as coffee) have produced an acute improvement in flow mediated dilatation,[6] suggesting that many who regularly consume such beverages frequently might gain at least a modest health benefit long term.

Although free and conjugated 3′-methoxy-4′-hydroxycinnamic acid (ferulic acid) does occur in the diet free or as glycosides, the dominant dietary source for many people will be conjugated 3′,4′-dihydroxycinnamic acid (caffeic acid) subsequently methylated by catechol-*O*-methyl transferase (COMT EC 2.1.1.6). COMT methylation of 3′,4′-dihydroxycinnamic acid (caffeic acid) can produce 3′-methoxy-4′-hydroxycinnamic acid (ferulic acid) and 3′-hydroxy-4′-methoxycinnamic acid (isoferulic acid) as shown in **Figure 1**. However, Kerimi et al.[2] did not address how variation in methylation regio-chemistry might impact on the yield of 3′-methoxycinnamic acid-4′-sulfate, although such potential is clear. From a xenobiotic metabolism perspective, 4′-methylation of *vic*-diols is usually considered the preferred mammalian strategy because it inhibits not only the formation of the *ortho*-quinone but also the formation of the *para*-quinone-methide, this latter a species often associated with drug toxicity through its ability to form adducts with proteins via Michael addition to *N*- or *S*-containing nucleophiles.

Clifford et al.[7] commented that the data for the regio-specificity of COMT methylation of 3′,4′-dihydroxycinnamic acid in vitro is extremely variable, some studies reporting a preference for 3′-methylation and others a preference for 4′-methylation. Moreover, studies in which volunteers consumed caffeoylquinic acid-rich beverages (such as coffee and maté) were not particularly helpful because both contained pre-existing conjugated 3′-methoxy-4′-hydroxycinnamic acid (*ca* 4% and *ca* 10% to *ca* 16% of total acyl-quinic acids in maté and in coffee, respectively),[8–11] and any 4′-sulfate produced therefrom inflates the yield derived from COMT 3′-methylation of 3′,4′-dihydroxycinnamic acid and distorts the 3′-/4′-methylation ratio.[1] In contrast, apples and associated products provide significant amounts of conjugated 3′,4′-dihydroxycinnamic acid (5-*O*-caffeoylquinic acid) with only trace amounts of pre-existing 3′-methoxy-derivatives (rarely reported and less than *ca* 0.2% of 3′,4′-dihydroxycinnamic acid derivatives in this study). A portion of the cinnamic acid metabolites will be hydrogenated to the corresponding 3-(phenyl)propanoic acids either by the gut microbiota or by the liver, and it is possible that some 3-(3′,4′-dihydroxyphenyl)propanoic acid will be produced by the gut microbiota catabolism of the flavan-3-ols and proanthocyanidins in the apple, but it is not a major product;[12] and because its methylation would be no different from 3-(3′,4′-dihydroxyphenyl)propanoic acid produced from 3′,4′-dihydroxycinnamic acid, this possibility is not an impediment to the study.[12]

Accordingly, the aim of this paper is to address the uncertainties surrounding the variation in 3′- and 4′-methylation of 3′,4′-dihydroxycinnamic acid and its impact on the yield of 3′-methoxycinnamic acid-4′-sulfate, by examining data from a study where volunteers consumed white-fleshed apple (WFA) or red-fleshed apple (RFA) snacks providing a dietary source of 5-*O*-caffeoylquinic acid (which is present in the flesh and in the peel of the apple). Then, the excreted 3′-methoxy metabolites and 4′-methoxy metabolites were determined in 24-h urine samples after an acute intake of RFA or WFA snacks. Afterwards, the same volunteers performed a second acute intake after 6 weeks of a sustained intake with WFA or RFA, and then the obtained results were compared.

This paper uses the metabolite nomenclature as recommended by Kay et al.[13] and IUPAC numbering for the acyl-quinic acids,[14] and all cited literature has been adjusted as necessary.

2. Experimental Section

2.1. Chemicals and Reagents

The standards used for the quantification of phenolic compounds in the WFA and RFA snacks were reported in our

previous studies.[15–17] Briefly, the standards used for the quantification of the 3′,4′-dihydroxycinnamic acid conjugate and its generated metabolites were as follows: 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3′-hydroxy-4′-methoxycinnamic acid, and 5-*O*-caffeoylquinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA); and 4′-hydroxy-3′-methoxycinnamic acid were from Fluka (Buchs, Switzerland).

Methanol (HPLC grade), acetonitrile (HPLC grade), and acetic acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water used was Milli-Q quality (Millipore Corp, Bedford, MA, USA). A stock solution of each phenolic compound was prepared by dissolving each standard compound in methanol at a concentration of 1000 mg L^{-1} and storing these in dark flasks at 4 °C. Stock dilutions were prepared daily.

2.2. White-fleshed and Red-fleshed Apples

In this study, two different apple varieties were used: a) the red-fleshed "Redlove" apple variety (RFA), which is a new genotype naturally biofortified in anthocyanins; and b) the common white-fleshed Granny Smith apple variety (WFA). Both apples were provided by NUFRI SAT (Mollerussa, Lleida, Spain). To preserve the phenolic compounds of the apples, a freeze-dried snack was prepared and provided to the volunteers. The detailed preparation process of the freeze-dried apple snacks is reported in our previous study.[16]

The chromatographic determination of phenolic compounds in both apples was performed with a fine powder of the freeze-dried snack samples obtained with the aid of an analytical mill (A11, IKA, Germany). The detailed phenolic composition of the WFA and RFA snacks is shown in **Table 1**.

2.3. Study Design: Acute Intake Studies in Humans

The studied population in the present study consisted of a subsample of the AppleCOR study, a parallel, randomized controlled trial conducted in 121 hypercholesterolemic subjects combining acute and sustained dietary interventions to study the cardiometabolic effects of RFA compared to common WFA. Participants from AppleCOR study consumed 80 g day^{-1} of the corresponding apple snack for 6 weeks. Nested within the sustained consumption study, a subsample of volunteers performed two acute studies, one at baseline and the other one after 6 weeks, consuming all at once 80 g of RFA or WFA snacks. In the present work, we specifically examine the 24-h urinary excretion of 3′-methoxy-4′-hydroxycinnamic acid and 3′-hydroxy-4′-methoxycinnamic acid and their associated metabolites in fourteen subjects that performed both acute postprandial studies consuming RFA (nine subjects) or WFA (five subjects). **Figure 2** shows a schematic representation of the subsample study design. The studied subjects were nine females, and five males, aged 25–66 years, with a body mass index (BMI) between 14.6 and 31.8 kg m^{-2}.

Inclusion criteria were to be aged >18, LDLc levels ≥115 mg dL^{-1} and willingness to provide informed consent before the initial screening visit. Exclusion criteria were: LDLc levels ≤115 and ≥190 mg dL^{-1}, and treatment with lipid-lowering drugs and

Table 1. Phenolic compounds (mg) consumed in 80 g of white-fleshed apple (WFA) snack, and 80 g of red-fleshed apple (RFA) snack, by the volunteers.

Phenolic compounds	WFA snack [mg 80 g^{-1} snack]	RFA snack [mg 80 g^{-1} snack]
Cyanidin arabinoside	0.85 ± 0.25	3.41 ± 0.12
Cyanidin galactoside	2.16 ± 0.55	30.9 ± 0.50
Anthocyanins	*3.01 ± 0.80*	*34.5 ± 0.43*
Gallic acid glucoside	1.34 ± 0.09	n.d.
Dihydroxyphenylacetic acid	0.35 ± 0.02	0.48 ± 0.07
Vanillic acid glucoside	0.86 ± 0.04	2.55 ± 0.32
Benzoic acids	*2.55 ± 0.03*	*3.03 ± 0.03*
Coumaric acid glucoside	5.49 ± 0.11	1.14 ± 0.05
Coumaric acid derivative	0.46 ± 0.03	n.d.
4′-hydroxy-3′-methoxycinnamic acid glucoside	0.29 ± 0.19	0.22 ± 0.11
4′-hydroxy-3′-methoxycinnamic acid derivative	0.07 ± 0.02	n.d.
5-*O*-caffeoylquinic acid	27.0 ± 1.13	69.0 ± 1.78
Hydroxycinnamic acids	*33.3 ± 2.35*	*70.4 ± 5.65*
Hydroxytyrosol	0.34 ± 0.02	n.d.
Phenyl alcohol	*0.34 ± 0.02*	*n.d.*
Catechin	7.77 ± 0.25	n.d.
Epicatechin	31.5 ± 0.47	4.93 ± 0.24
Dimer	70.4 ± 6.21	9.12 ± 0.43
Trimer	10.9 ± 1.01	n.d.
Tetramer	1.80 ± 0.12	n.d.
Flavan-3-ols	*121 ± 8.08*	*14.1 ± 0.67*
Quercetin	0.10 ± 0.02	n.d.
Dihydroquercetin	0.33 ± 0.17	0.10 ± 0.08
Quercetin arabinoside	5.92 ± 1.33	3.93 ± 0.42
Quercetin rhamnoside	5.72 ± 1.12	9.68 ± 1.61
Quercetin glucoside	5.71 ± 0.56	3.34 ± 0.34
Quercetin rutinoside	0.49 ± 0.52	0.16 ± 0.07
Quercetin diglucoside	0.09 ± 0.02	n.d.
Dihydrokaempferol	0.13 ± 0.11	0.24 ± 0.06
Dihydrokaempferol glucoside	n.d.	0.17 ± 0.06
Kaempferol rutinoside	0.25 ± 0.09	0.12 ± 0.06
Flavonols	*18.7 ± 3.94*	*17.7 ± 2.70*
Luteolin glucoside	0.07 ± 0.02	0.06 ± 0.05
Apigenin glucoside	0.01 ± 0.01	n.d.
Flavones	*0.17 ± 0.04*	*0.06 ± 0.05*
Naringenin glucoside	0.41 ± 0.00	0.55 ± 0.09
Eriodictyol glucoside	0.48 ± 0.05	0.80 ± 0.08
Flavanones	*0.88 ± 0.05*	*1.36 ± 0.17*
Phloretin glucoside	8.32 ± 2.70	21.4 ± 4.10
Phloretin xylosyl glucoside	10.8 ± 0.91	29.4 ± 5.82
Hydroxyphloretin xylosyl glucoside	0.80 ± 0.14	1.24 ± 0.19
Dihydrochalcones	*19.9 ± 3.76*	*52.0 ± 10.1*
TOTAL PHENOLICS	*197 ± 17.5*	*193 ± 16.5*

The results are expressed as the mean ± standard deviation ($n = 6$).

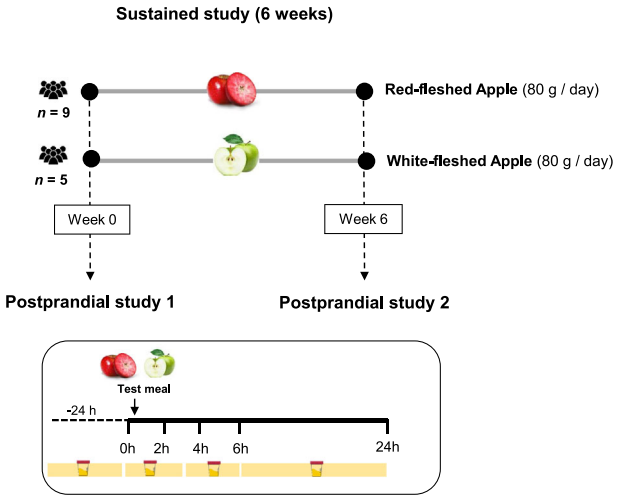

Figure 2. Study design of two postprandial apple snack intakes

functional foods; treatment for prediabetes or diagnosed type 1 and type 2 diabetes mellitus; BMI ≥35 kg m^{-2}; triglyceride levels ≥350 mg dL^{-1}; anemia (hemoglobin ≤13 g dL^{-1} in men and ≤12 g dL^{-1} in women); subjects diagnosed with intestinal disorders such as chronic disease, colitis ulcerous, celiac disease, and irritable bowel syndrome; presentation with fructose and/or sorbitol and/or gluten intolerance; use of antioxidants supplements; pregnant or intending to become pregnant; to be in breast-feeding period; chronic alcoholism; smoking; current or past participation in a clinical trial or consumption of a research product in the 30 days prior to inclusion in the study; and failure to follow the study guidelines.

Participants signed informed consent prior to their participation in the study, which was approved by the Clinical Research Ethical Committee of Institut d'Investigació Sanitària Pere Virgili (Reus, Spain) (S033/04Nov2016). The protocol and trial were conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (GCP ICH) and were reported as CONSORT criteria. The trial was registered at Clinical Trials.gov Identifier: NCT03795324.

During the sustained intervention, participants were instructed to completely refrain from consuming anthocyanins-rich foods (berries, grapefruit, plums, figs, pomegranate, green and red apples, avocado, black olives, red and black beans, red wine, and mushrooms), and limit the consumption of coffee to one or two cups. Participant who performed the first acute intake study were instructed to maintain a polyphenol-free diet the two days prior to the study (vegetables and fresh fruits, pulses, virgin olive oil (VOO), species, wine, coffee, tea, chocolate, etc).

During the postprandial studies, volunteers stayed from 08:00 a.m. to 02:00 p.m., and received a light meal before leaving. Urine samples were collected 24 h before the intake, and 24 h after the test products intake. The total volume of urine of each volunteer was measured before storing the aliquots at −80 °C in the central laboratory's Biobanc of HUSJ-Eurecat until required. For the phenolic metabolite chromatographic analysis, each biological sample was analyzed and pre-treated in duplicate.

2.4. Dosage Information

The phenolic dose administered to participants through the apple snacks during the sustained (6 weeks) and the acute studies (24 h) was 197 mg 80 g^{-1} WFA snack day^{-1}, and 193 mg 80 g^{-1} RFA snack day^{-1}. Regarding to the hydroxycinnamic acids, which was the aim of this study, the dose administered was 33.3 mg 80 g^{-1} WFA snack day^{-1}, and 70.4 mg 80 g^{-1} RFA snack day^{-1}, and these were mainly provided with 5-*O*-caffeoylquinic acid (Table 1).

2.5. Urine Sample Pre-treatment

Prior to the chromatographic analysis, urine samples were subjected to a clean-up with micro-Elution solid-phase extraction (μSPE) by using OASIS HLB (2 mg, Waters, Milford, MA, USA) micro-cartridges.[16,17] Briefly; the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. Then, 100 µL of phosphoric acid at 4% was added to 100 µL of the human urine sample, and the resultant solution was loaded into the micro-cartridge. The retained phenolic metabolites were then eluted with 2 × 50 µL of methanol.

2.6. Ultra-performance Liquid Chromatography Coupled to Tandem Mass Spectrometry (UPLC-ESI-MS/MS)

The analysis of phenolic compounds in the apple snacks, and in urine samples was performed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) as the detection system (Waters, Milford, MA, USA), and the used conditions were the reported in our previous study.[15–17] Supplemental Table 1 (Supporting Information) shows the selected reaction monitoring (SRM) transition for quantification, as well as the cone voltage and collision energy for the 3′,4′-dihydroxycinnamic acid conjugate in apple snacks and its generated metabolites in urine samples. Due to the lack of commercial phenolic standards and their generated metabolites, the 3′-methoxy and 4′-mehoxy metabolites were tentatively quantified by using the calibration curve of 4′-hydroxy-3′-methoxycinnamic acid, and 3′-hydroxy-4′-methoxycinnamic acid, respectively (see Supplemental Table 1, Supporting Information).

2.7. Statistical Analysis

The results are presented as mean values ± standard deviation (SD) for the phenolic compounds in WFA and in RFA snacks; and mean values ± standard error of the mean (SEM) for the excreted phenolic metabolites in 24-h urine samples. The quotient (largest / smallest) is used to better demonstrate the variability of data that are not normally distributed.

3. Results and Discussion

3.1. Variation in the Methylation of 3′,4′-dihydroxycinnamic Acid After Acute Apple Ingestion

The objective of this study is to determine for dietary 3′,4′-dihydroxycinnamic acid, consumed as 5-*O*-caffeoylquinic acid in

Table 2. Concentration (μmol) of 3′,4′-dihydroxycinnamic acid metabolites excreted in urine samples (24 h) after a postprandial intake of red-fleshed apple snack (RFA), and white-fleshed apple (RFA) snack intakes in 14 healthy volunteers.

Phenolic compounds	RFA									WFA					Mean±SEM	Largest / Smallest
	Male	Female	Male	Female	Female	Female	Female	Female	Male	Female	Male	Female	Male	Female		
	59 y	62 y	65 y	25 y	56 y	66 y	41 y	33 y	55 y	63 y	40 y	60 y	61 y	35 y		
	V1_R	V2_R	V3_R	V4_R	V5_R	V6_R	V7_R	V8_R	V9_R	V10_W	V11_W	V12_W	V13_W	V14_W		
(C_6-C_3)																
3′-methoxy metabolites																
3′-methoxy-4′-hydroxycinnamic acid	0.23	0.11	0.12	0.05	0.15	n.d.	0.01	0.03	n.d.	0.03	0.11	0.11	n.d.	0.02	0.07 ± 0.02	7.67
3′-methoxycinnamic acid-4′-sulfate	13.8	23.3	6.11	4.38	4.46	n.d.	3.69	0.11	0.11	n.d.	19.8	7.84	2.31	4.85	6.49 ± 1.99	212
3′-methoxycinnamic acid-4′-glucuronide	0.22	0.43	0.28	0.10	0.18	0.04	0.21	0.04	n.d.	n.d.	0.49	0.16	0.01	0.23	0.17 ± 0.04	49.0
3′-methoxy-4′-hydroxycinnamoyl-glycine	1.39	1.86	0.55	0.65	0.05	n.d.	0.31	0.02	n.d.	n.d.	1.21	1.36	0.74	0.05	0.59 ± 0.17	93.0
3-(3′-methoxy-4′-hydroxyphenyl) propanoic acid	3.09	1.68	0.10	0.79	n.d.	n.d.	0.42	0.18	n.d.	n.d.	0.97	0.57	1.41	0.29	0.68 ± 0.24	30.9
3-(3′-methoxyphenyl) propanoic acid-4′-sulfate	11.1	9.10	1.47	5.59	1.92	n.d.	1.93	0.56	0.22	n.d.	9.50	4.45	n.d.	4.76	3.33 ± 1.05	50.4
3-(3′-methoxyphenyl) propanoic acid-4′-glucuronide	0.82	2.09	0.19	0.27	0.01	n.d.	0.14	0.33	0.01	n.d.	0.27	0.11	0.09	0.05	0.31 ± 0.15	209
Sum	*30.6*	*38.5*	*8.84*	*11.8*	*6.73*	*0.04*	*6.71*	*1.27*	*0.34*	*0.03*	*32.4*	*14.6*	*4.57*	*6.26*	*11.6 ± 3.45*	
4′-methoxy metabolites																
3′-hydroxy-4′-methoxycinnamic acid	3.70	0.35	0.08	0.17	0.21	0.29	0.12	0.20	0.12	n.d.	0.03	0.21	0.01	0.15	0.40 ± 0.26	239
4′-methoxycinnamic acid-3′-sulfate	0.67	2.06	0.56	0.44	0.62	n.d.	0.82	0.27	n.d.	0.02	1.64	1.31	0.96	0.26	0.69 ± 0.17	7.82
4′-methoxycinnamic acid-3′-glucuronide	0.45	0.88	0.51	0.33	0.34	0.25	0.48	0.03	n.d.	0.00	1.08	0.30	0.27	n.d.	0.35 ± 0.09	28.5
3′-hydroxy-4′-methoxycinnamoyl-glycine	1.57	0.20	0.10	0.88	0.10	n.d.	0.11	0.06	n.d.	n.d.	1.66	0.47	n.d.	0.10	0.37 ± 0.15	26.7
3-(4′-methoxy-3′-hydroxyphenyl) propanoic acid	0.06	0.19	0.01	3.28	0.00	0.02	0.17	0.73	0.33	6.74	0.11	0.07	0.01	0.37	0.86 ± 0.51	212
3-(4′-methoxyphenyl) propanoic acid-3′-sulfate	2.47	15.7	2.89	12.8	14.1	n.d.	0.20	1.03	n.d.	9.59	1.46	1.26	n.d.	n.d.	4.40 ± 1.57	77.7
3-(4′-methoxyphenyl) propanoic acid-3′-glucuronide	1.01	5.99	0.20	2.75	1.85	n.d.	0.03	0.33	n.d.	2.47	0.90	0.10	n.d.	0.05	1.12 ± 0.45	193
Sum	*9.92*	*25.4*	*4.36*	*20.7*	*17.3*	*0.56*	*1.92*	*2.66*	*0.45*	*18.8*	*6.87*	*3.72*	*1.26*	*0.94*	*8.20 ±2.31*	
Methylation ratio (3′-/4′-methylation)	3.09	1.52	2.03	0.57	0.39	0.07	3.49	0.48	0.76	0.004	4.71	3.93	3.63	6.68	*2.24 ± 0.55*	

n.d., not detected.

apple snacks, the relative importance of 3′- or 4′-methylation by COMT, and the extent to which this influences the excretion of 3′-methoxycinnamic acid-4′-sulfate. Apple snacks were chosen for this study because they supply significant 3′,4′-dihydroxycinnamic acid with only traces of 3′-methoxy-4′-hydroxycinnamic acid and do not contain 3′-hydroxy-4′-methoxycinnamic acid (Table 1), which would otherwise impede calculation of the 3′ / 4′-methylation ratio.

To carry out this study, five volunteers (two males and three females aged 35–63 years) consumed a WFA snack delivering 76 ± 3 μmol 5-*O*-caffeoylquinic acid and no more than *ca* 0.1 μmol of 3′-methoxy-4′-hydroxycinnamic acid derivatives. Nine volunteers (three males and six females aged 25–66 years) consumed an RFA snack delivering 195 ± 5 μmol 5-*O*-caffeoylquinic acid and not more than 0.07 μmol 3′-methoxy-4′-hydroxycinnamic acid derivatives.

Table 2 presents the 24-h postprandial excretion of seven 3′-methoxycinnamic acid metabolites and seven 4′-methoxycinnamic acid metabolites, plus the sum for each category, and the 3′- / 4′-methylation ratio for each volunteer. The relevant mean and SEM are also presented, but in every case the standard error is so large that it implies impossible negative values, and it is clear that the data are extremely variable and far from normally distributed. For that reason, we also express the variability as the quotient (largest / smallest).

From the obtained results, only one male (V9) had a 3′ / 4′-methylation ratio below 1 whereas five out of nine females did, of whom V6 achieved 93% 4′-methylation and V10 >99% methylation. In contrast, female V14 produced over 90% 3′-methylated metabolites. It has been reported that COMT expression in the endometrium varied during the menstrual cycle, being down regulated in the mid-secretory phase and up-regulated in the proliferative phase, and if this applies to other tissues, it could be a factor contributing to the more variable behavior of the female volunteers.[18]

In a second postprandial study, the equivalent data are available for 11 of the 14 volunteers who participated in the first postprandial study. **Table 3** shows these results and it can be observed how eight volunteers maintained their previous pattern of methylation. Of these eight, seven showed only a modest change not exceeding ±7% in the percentage of 3′-methylated metabolites excreted. The eighth, female V5, changed from 28% to 40% 3′-methylation, approaching a two-fold change in the methylation ratio (V5 0.39; 0.68) (see **Table 4**).

The remaining three volunteers, one female (V4: 36.3% to 64.43%) and one male (V9: 43.0% to 88.3%) changed from favoring 3′-methylation to favoring 4′-methylation; and one male (V3: 67.0% to 11.3%) changed from favoring 4′-methylation to favoring 3′-methylation (Table 3 and Table 4). Unfortunately, female volunteers V6, V8, and V10 did not provide a urine sample in the second study, but most of the females whose urines were analyzed V2 (60.3% to 57.1%), V7 (77.8% to 72.7%), V12 (79.7% to 83.5%), and V14 (86.9% to 83.0%) barely changed the percentage of 3′-methylated metabolites excreted (Table 4). While these results are consistent with a modulating effect of the menstrual cycle, it clearly cannot be the only factor controlling COMT regiochemistry, and there may be a role for COMT polymorphisms.[19]

Two female volunteers (V6, V10) who strongly favored 4′-methylation did not excrete either of the 3′-methylated sulfates, whereas females V4, V5 and V8 excreted both, although the hydrogenated metabolite dominated for V4 and V8. For eight volunteers (four female, four male) the potentially beneficial 3′-methoxycinnamic acid-4′-sulfate was the dominant 3′-methylated metabolite, and for the other male it was 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate. This suggests that SULT1E1 conjugation with sulfate is not a limiting factor if 3′-methylation has occurred,[20] but that hydrogenation before or after absorption is an important determinant of the yield of 3′-methoxycinnamic acid-4′-sulfate.

Table 3. Methylation ratio after a first postprandial intake (24-h urine), and a second postprandial intake (24-h urine) after 6 weeks of WFA, and RFA snacks consumption.

Volunteer	Sex	Age	3′- / 4′- methylation ratio	
			First postprandial	Second postprandial
V1_R	M	59	3.08	2.74
V2_R	F	62	1.52	1.33
V3_R	M	65	2.03	0.13
V4_R	F	25	0.57	1.81
V5_R	F	56	0.39	0.68
V6_R	F	66	0.07	–
V7_R	F	41	3.49	2.66
V8_R	F	33	0.48	–
V9_R	M	55	0.76	7.58
V10_W	F	63	0.004	–
V11_W	M	40	4.72	2.54
V12_W	F	60	3.92	5.08
V13_W	M	61	3.63	6.53
V14_W	F	35	6.66	4.88

Table 4. Percentage (%) of 3′-methylated metabolites excreted in 24h-urine and its change (%) after a first postprandial intake, and a second postprandial intake after 6 weeks of WFA and RFA snacks consumption.

Volunteer	Sex	Age	Percentage (%) of 3′-methylated metabolites excreted		Change (%)
			First postprandial	Second postprandial	
V1_R	M	59	75.5	73.3	1.50
V2_R	F	62	60.3	57.1	2.67
V3_R	M	65	67.0	11.3	71.2
V4_R	F	25	36.3	64.4	−27.9
V5_R	F	56	28.0	40.3	−18.0
V6_R	F	66	6.67	–	–
V7_R	F	41	77.8	72.7	3.35
V8_R	F	33	32.3	–	–
V9_R	M	55	43.0	88.3	−34.5
V10_W	F	63	0.16	–	–
V11_W	M	40	82.5	71.7	6.99
V12_W	F	60	79.7	83.5	−2.36
V13_W	M	61	78.4	86.7	−5.05
V14_W	F	35	86.9	83.0	2.32

The 14 C_6–C_3 metabolites excreted accounted for between 0.2 and 48.5% of the 3′,4′-dihydroxycinnamic acid consumed, and the fate of the remainder is unclear. Gut microbiota 4′-dehydroxylation probably accounts for the major loss, but loss in urine as acyl-quinic acid conjugates and loss in feces and β-oxidation could also contribute.[21,22]

4. Concluding Remarks

It can be concluded that these results do not support the generally accepted dogma that *vic*-diols such as 3′,4′-dihydroxycinnamic acid are predominantly 4′-methylated because only six out of 14 volunteers favored this route, and for only two did 4′-methylation account for at least 90% of the C_6–C_3 metabolites excreted in 24 h. However, it is clear, that whether 3′- or 4′-methylation dominates can change over time. While the menstrual cycle might be a factor, such a reversal was also shown by one male volunteer. Clearly, the yield of the potentially beneficial 3′-methoxycinnamic acid-4′-sulfate is extremely variable, ranging from undetectable to 71% of the total C_6–C_3 metabolites excreted (zero to 26% of the 3′,4′-dihydroxycinnamic acid consumed). With such dihydroxy substrates 3′-methylation is an absolute prerequisite for the production of 3′-methoxycinnamic acid-4′-sulfate, and the extent of hydrogenation before or after absorption is also a crucial determinant.

To unravel the complexities, further studies are needed in which volunteers consume first ^{13}C-labeled 3′,4′-dihydroxycinnamic acid and second ^{13}C-labeled 3′-methoxy-4′-hydroxycinnamic acid at weekly intervals over 2 months / two menstrual cycles and collect 24-h urines for detailed LC-MS analysis. Volunteers should keep detailed diet diaries, be genotyped for COMT polymorphisms and consider their gut microbiota characteristics.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.R.: Methodology; Writing-review and editing; Validation, and Supervision; R.S.: Validation; Supervision; M.P.R.: Validation; Supervision; M.J.M.: Validation; Supervision; M.N.C.: Conceptualization, Supervision, writing-review and editing; A.M.: Investigation; writing – original draft; Supervision.

Data Availability Statement

Data available on request from the authors.

Keywords

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