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Urinary Excretion of Ecdysterone and Its Metabolites Following Spinach Consumption

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Scope: The phytosteroid ecdysterone is present in spinach. In this study, the urinary elimination of ecdysterone and its metabolites in humans is investigated following spinach consumption of two different culinary preparations.

Methods and results: Eight participants (four males, four females) ingested 950 (27.1) g sautéed spinach (average [±standard deviation (SD)]) and 912 (70.6) g spinach smoothie as second intervention after washout. Post-administration urines are analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). After intake of both preparations, ecdysterone and two metabolites, 14-deoxy-ecdysterone, and 14-deoxy-poststerone, are excreted in urine. The maximum concentration of ecdysterone is ranging from 0.09 to 0.41 μg mL^{-1} after sautéed spinach and 0.08–0.74 μg mL^{-1} after smoothie ingestion. The total excreted amount (mean% [±SD]) in the urine as a parent drug plus the metabolites is only 1.4 (1.0) for both sautéed spinach and smoothie. The apparent sex related differences in 14-deoxy-poststerone excretion will need further investigations.

Conclusion: Only a small proportion of ecdysterone from spinach is excreted into urine. No significant differences are found in concentration and recovered amount (%) of ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone in urine between sautéed spinach and smoothie ingestion. A discrimination between ecdysterone from food or preparations will be challenging based on urinary concentrations only, at least for later post-administration samples.

1. Introduction

Spinach (*Spinacia oleracea*), a vegetable commonly consumed in human diet worldwide, is one of the well-known crop sources of ecdysteroids. Its ecdysteroid content has already been reported in the 1980s and was widely studied since then.[1–4] Ecdysterone is the most abundant ecdysteroid found in spinach, together with several minor ecdysteroids such as polypodin B, ecdysone, 2-deoxy-ecdysterone, and makisterone A.[4–7] In spinach leaves, the levels of ecdysterone are reported from 4 to 800 μg g^{-1} fresh weight (f.w.), and similarly from 17 to 885 μg g^{-1} dry weight displaying significant variations due to season of harvest, development phase, geographical location, natural environment as well as plant variety.[2–10] Abnormally, Grucza et al. have reported ecdysterone amount in fresh spinach as 0.1 μg g^{-1} f.w.[11] The levels of ecdysterone reported in spinach suggest that the normal amount of ecdysterone from spinach in a daily diet is rather low.

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Ecdysteroids have long been recognized for various potential beneficial effects on animal species and humans, including hypolipidemic, antidiabetic, hepatoprotective, adaptogenic as well as growth-promoting and anabolic effects.[12–21] In vivo and in vitro studies have shown that ecdysterone increases protein synthesis and muscle mass.[20,21] As reported in Parr et al.,[21] this effect involves mediation by estrogen receptor beta (ER-β). In silico data supported the hypothesis that ecdysterone binds to ER-β and thus stimulates the anabolic activity without causing androgen related side effects.[20–22]

Traditionally, spinach had the reputation for boosting muscle power promoted by the fictional cartoon character, Popeye. The anabolic effect of its ecdysteroid content may offer a rational basis for this folklore theory.[7,23] Due to its anabolic properties, ecdysterone has long raised attention as a "natural alternative" performance enhancer in sports. An increasing number of dietary supplements containing ecdysterone from spinach and other plant extracts are available and advertised to increase strength and muscle mass, reduce fatigue, and ease recovery.[20] The recommended doses for sportsmen are up to 1000 mg day^{-1} while even a massive spinach consumption (1 kg day^{-1}) can rarely exceed 100 mg of ecdysterone intake per day from this source.[23–25] In a recently reported study, a controlled administration in humans with long-term administration of an ecdysterone-containing supplement demonstrated an increase in muscle mass and physical power.[19] Following these results and considering the given literature for its anabolic activity, ecdysterone has been included in the Monitoring Program of the World Anti-Doping Agency (WADA) since 2020 under the section "Anabolic Agents, In-and Out-of-Competition."[26] Similarly to the results in young men, a recently published study reports an increase of skeletal muscle fitness after spinach extract administration for 12 weeks in adults over 50 years of age.[18]

In previous studies, the urinary elimination and metabolism of ecdysterone were reported following the administration of ecdysterone supplements or pure ecdysterone to humans.[27–30]

Concerning an application in doping control analysis, the urinary elimination and metabolism of ecdysterone were reported following the administration of ecdysterone supplements or pure ecdysterone in humans.[29–32] As spinach is worldwide considered as common in human diet, it is currently discussed which implications will result from an inclusion of its phytosteroid ingredient in the list of prohibited substances and methods of WADA. In the present study, two differently processed spinach preparations were administered to humans to investigate the urinary elimination of ecdysterone and its metabolites following spinach consumption of two different preparations and to compare it with the already reported pure ecdysterone administration.

2. Experimental Section

2.1. Chemicals

Reference material of ecdysterone (2β,3β,14α,20β,22R,25-hexahydroxy-5β-cholest-7-en-6-one, purity > 95%) was purchased from Steraloids (Newport, RI, USA). Ponasterone (2β,3β,14α,20β,22R-pentahydroxy-5β-cholest-7-en-6-one) used as internal standard (ISTD), was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Alpha-14-deoxy-ecdysterone and alpha-14-deoxy-poststerone were purchased from Extrasynthese (Genay CEDEX, France). Stock solutions of the analytes in methanol were prepared at a concentration of 1 mg mL^{-1} and stored at −20 °C until further use.

2.2. Administration Studies

2.2.1. Study Design

The study was approved by the ethical committee of the German Sport University Cologne (no. 152/2020) and carried out following the regulations of the Declaration of Helsinki. Eight healthy subjects, four females and four males, participated in two different administration studies. The participants were aged 27 (3) years, height 174 (9) cm, and weighed 76 (15) kg. All participants were instructed to avoid consuming other food that contains ecdysterone before and during the studies. For each study, blank urine was collected imminently prior to administration. Post-administration urine samples were collected for 72 h, i.e., 3 days. Sampling time and urine volume were recorded. Aliquots of urine samples were stored frozen at −18 °C until analysis.

2.2.2. Food Preparation

Frozen spinach was purchased from the German local market (Globus, Cologne, Germany, lot: L21263-LN04). On the day of administration, frozen spinach was thawed and cooked with low heat for 20–25 min and administered as sautéed spinach. For the spinach smoothie, the sautéed spinach was transferred to a blender and was agitated until smooth. Each participant always received a portion which corresponded to one package of frozen spinach. Each package was labeled to contain 1 kg of spinach. Before the intake, each portion was weighed and recorded.

2.2.3. Dosage Regimen

All participants received sautéed spinach leaves as first intervention. After 1 week washout period, all participants ingested a smoothie made of sautéed spinach leaves as second intervention. The mean (standard deviation (SD)) portion of sautéed spinach consumed by each volunteer was 950 (27.1) g, while for the spinach smoothie it was 912 (70.6) g.

2.3. Sample Preparation

2.3.1. Food Analysis

The sautéed spinach or smoothie was weighted (10 g) into a 50 mL conical tube, ethanol:water (80:20, v:v) was added to a final volume of 35 mL. The mixture was homogenized with Ultra-Turrax T 25 basic (IKA WERKE, Staufen, Germany) for 3 min at 19.000–24.000 min^{-1} and centrifuged with Universal 320R (Hettich, Tuttlingen, Germany) at 3011 RCF for 10 min. The supernatant was collected and the residue was re-extracted under the

same conditions. In total, the same procedure was repeated three times to ensure the maximum extraction of ecdysterone. The extracts were combined and concentrated under vacuum at 60 °C using a rotary evaporator. The dry extract was reconstituted with 100 mL methanol, diluted with methanol (1:50, v:v), and filtered through a particle filter (0.2 µm syringe filters). Afterwards, 80 µL of the diluted spinach samples were spiked with 20 µL ISTD ponasterone (working solution 1000 ng mL^{-1}) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Three replicates of sautéed spinach and smoothie, each, were prepared and analyzed.

2.3.2. Urine Analysis

The urine samples were prepared as reported previously.[27] In brief, urine samples (200 µL) were spiked with 10 µL of ISTD ponasterone (10 µg mL^{-1}) and diluted to 1 mL with MeOH:H_2O (10:90, v/v). After vortex-mix and centrifugation at 9677 RCF for 8 min, 5 µL of the supernatants were injected into the LC-MS/MS system for analysis.

2.4. Instrumentation

The instrumental analyses were performed by ultrahigh performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) system consisting of an Agilent 1290 Infinity II UHPLC coupled to an Agilent 6495 triple quadrupole tandem MS (Agilent Technologies GmbH, Waldbronn, Germany), utilizing an Agilent Jet Stream electrospray ionization (ESI) source and ion funnel adapted from the earlier published procedure.[27] For quantitation of ecdysterone in spinach and ecdysterone and its metabolites in urine samples the same LC-MS/MS method was used, however using a different LC column and flow rate of the mobile phase. Details including exemplary chromatograms are presented in the supplemental material.

2.5. Software

MassHunter software version 10 from Agilent was used for data acquisition, MassHunter Quant version 10 from Agilent was used for data processing. Microsoft Excel 365 (Microsoft, Munich, Germany) and OriginPro, version 2019b (OriginLab Corporation, Northampton, MA, USA) were used for data analysis, statistical evaluation, and data visualization.

2.6. Evaluation of Urinary Data

The parameters of urinary excretion kinetics of ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone were evaluated as previously described.[27] In brief, the concentration of the three analytes were corrected with a dilution factor (1:5). The excretion rate (E_{rate}), the half-life, and the cumulative amount of three analytes were calculated according to Shargel and Yu,[33] as also previously described in detail.[27] The amount of analytes recovered in the urines were reported in percentage values in comparison with the individual intake of ecdysterone in each subject.

Table 1. Ecdysterone content (µg g^{-1}) f.w. from two different spinach preparations ($n = 3$).

Type of preparation	Mean (SD) [µg g^{-1}]
Sautéed spinach	20.2 (0.3)
Smoothie	20.0 (2.8)

2.7. Statistical Analysis

The anthropometric data of participants, the intake of food, and quantitation of ecdysterone in spinach was reported as mean (SD). The urinary elimination parameters such as maximum concentration (C_{max}), maximum excretion rate ($E_{rate\text{-}max}$), cumulative amount, recovered amount (%), and half-life were reported as a range (min – max), as mean (SD), and/or as median (IQR, interquartile range). Samples with $n = 3$ were assumed to be not normally distributed due to the low number of samples. Samples ($n > 3$) were tested with Saphiro-Wilk for normal distribution. The paired-sample *t*-test was used to evaluate the statistical difference for normally distributed data. The nonparametric Wilcoxon signed-rank test was used to evaluate the statistical difference for non-normally distributed data. A *p*-value <0.05 was considered significant.

3. Results and Discussion

3.1. Ecdysterone Amount in Spinach

Analysis of ecdysterone in sautéed spinach and smoothie, both, showed an ecdysterone content of 20 µg g^{-1} f.w. The results of ecdysterone determination in spinach from these two preparations are reported in **Table 1**. Based on the serving dose of each participant, the average (SD) intake of ecdysterone for sautéed spinach and smoothie were 19.3 (0.5) and 18.2 (1.4) mg, respectively.

3.2. Urinary Excretion Parameters of Ecdysterone and Its Metabolites

The quantitation of ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone in the urine samples was performed by LC-MS coupled to triple-quadrupole applying the previously validated method.[27] Details of the method as well as exemplary chromatograms are available in the supplemental material.

Blank urine collected before the ingestion of spinach showed neither signals for ecdysterone nor for its metabolites in all participants. After the intake of ecdysterone from food, ecdysterone and its two metabolites, 14-deoxy-ecdysterone and 14-deoxy-poststerone, were found to be excreted in urine and quantified by comparison with the reference standards. After ingestion of sautéed spinach and smoothie, all eight participants excreted ecdysterone in urine.

The concentration over time (A), the excretion rate against midpoint time of sample collection (B), and the cumulative amount (C) after the intake of sautéed spinach (upper) and smoothie (bottom) for ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone are shown in **Figures 1–3**.

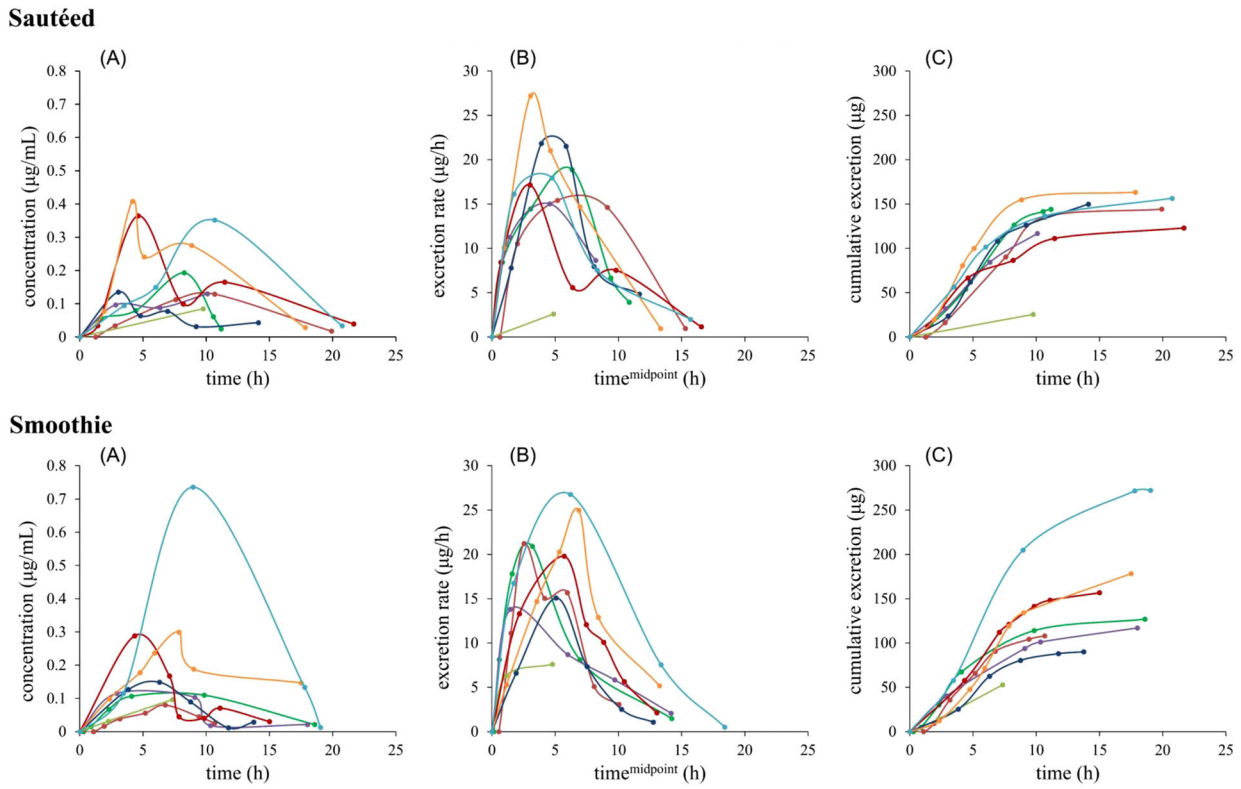

Figure 1. Urinary excretion kinetics of ecdysterone: concentration over time (A), excretion rate against midpoint time of sample collection (B), and cumulative amount (C) after the intake of sautéed spinach (upper) and smoothie (bottom). Each color represents one subject.

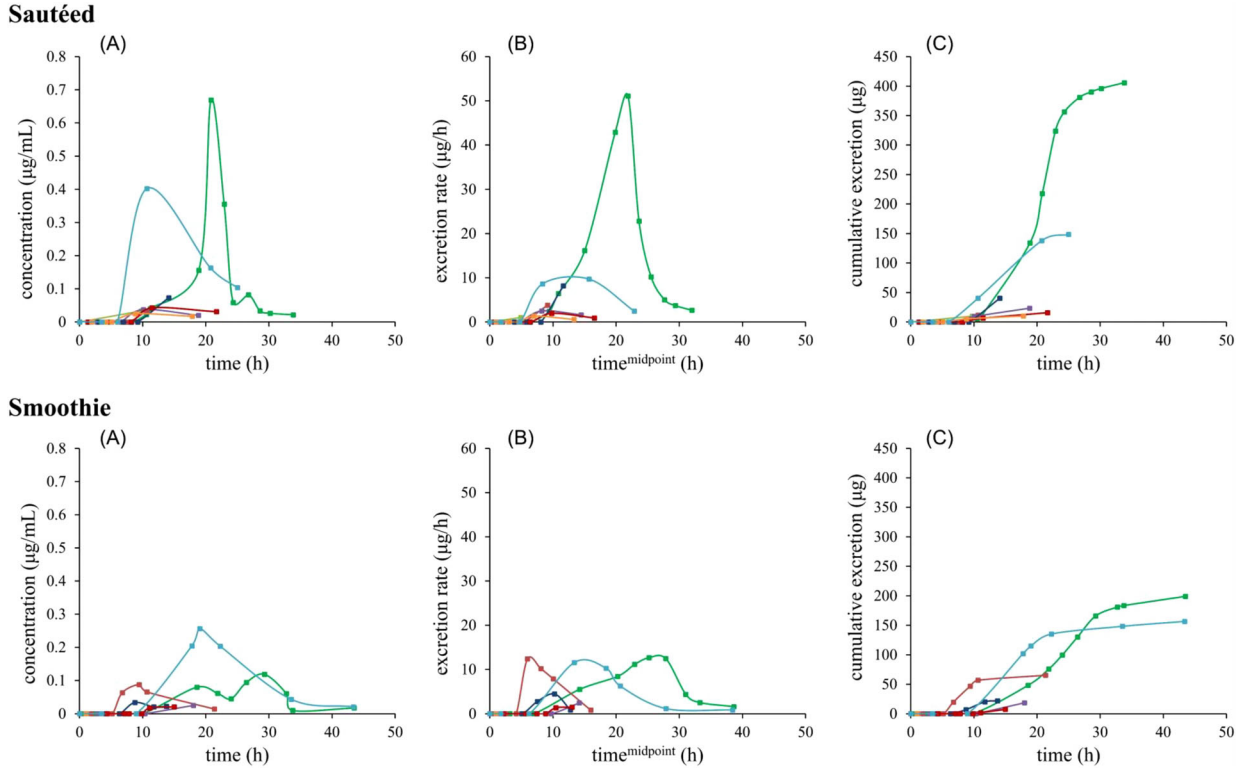

Figure 2. Urinary excretion kinetics of 14-deoxy-ecdysterone: concentration over time (A), excretion rate against midpoint time of sample collection (B), and cumulative amount (C) after the intake of sautéed spinach (upper) and smoothie (bottom). Each color represents each subject.

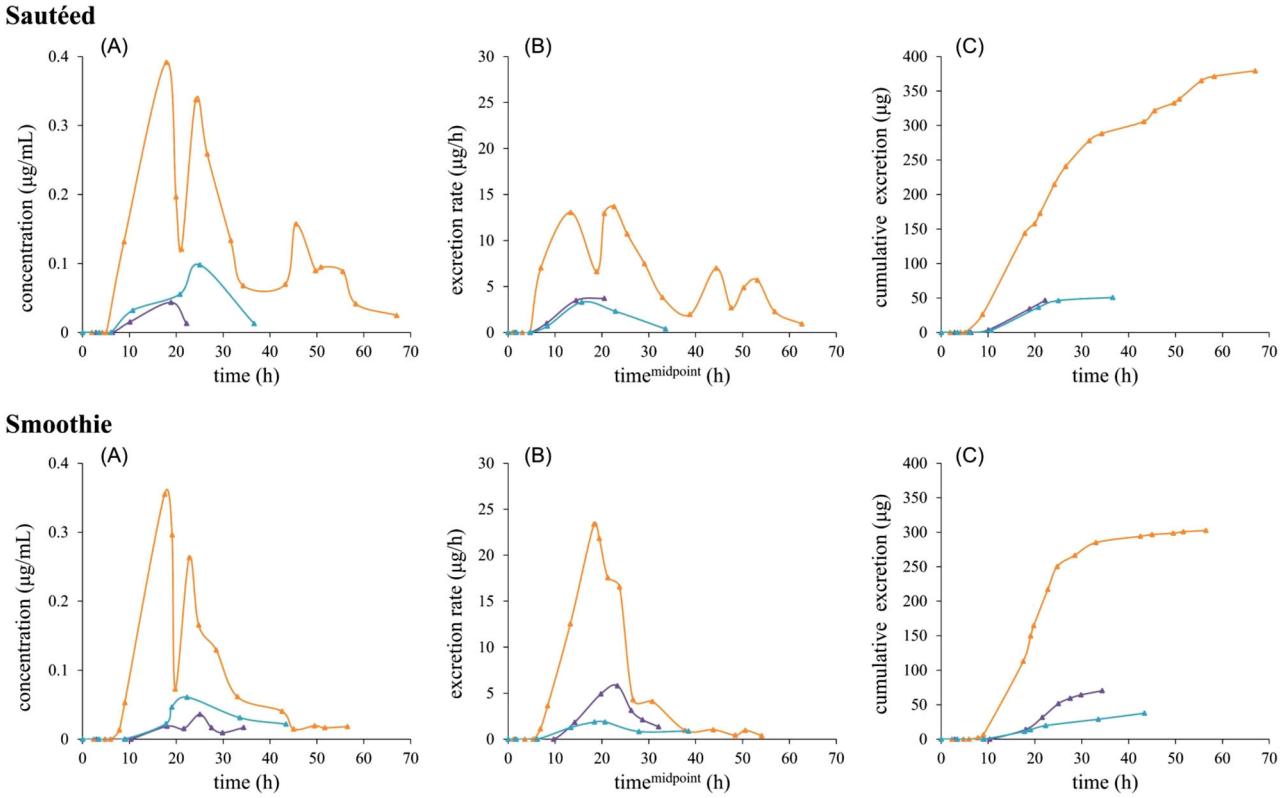

Figure 3. Urinary excretion kinetics of 14-deoxy-poststerone: concentration over time (A), excretion rate against midpoint time of sample collection (B), and cumulative amount (C) after the intake of sautéed spinach (upper) and smoothie (bottom). Each color represents each subject.

The maximum concentration of ecdysterone in urine was observed between 3 and 10 h after sautéed spinach intake and ranged between 0.09 and 0.41 µg mL^{-1}. After smoothie consumption, the maximum concentration was also observed at a similar time range and determined as 0.08–0.74 µg mL^{-1}. In comparison, the maximum urinary concentration of ecdysterone was between 4.4 and 30 µg mL^{-1} after 50 mg of pure ecdysterone.[27] In addition, in multiple dose administration of ecdysterone supplement, after intake of eight capsules a day (equivalent to 48 mg ecdysterone) for 10 weeks, the concentration of ecdysterone in urine was quantified in 18 of 19 samples with concentration ranges of ecdysterone as follow: not detected (n = 1), 0–0.05 µg mL^{-1} (n = 1), 0.05–0.1 µg mL^{-1} (n = 2), 0.1–0.25 µg mL^{-1} (n = 7), 0.25–0.5 µg mL^{-1} (n = 4), 0.5–0.75 µg mL^{-1} (n = 3), 0.75–1.5 µg mL^{-1} (n = 1) (unpublished data). Although urine samples during the multiple dose administration were only collected one time in week 5 and once after week 10, this information might be useful for anti-doping laboratories to consider a threshold limit for ecdysterone concentration in the urine if the ecdysterone source is from spinach.

In our previous study, ecdysterone was already detectable in urine after 45 min of intake of pure ecdysterone as an oral solution (c = 1.35 µg mL^{-1}).[27] After smoothie intake ecdysterone was detected and quantified after 53 min in one participant (c = 0.015 µg mL^{-1}), whereas in another participant ecdysterone was not detected in the 1-h post-administration urine (c < limit of detection (LOD)). This may indicate the interindividual variability in the absorption rate for each participant, which consequently also affects the earliest detection time of ecdysterone in urine. However, in most cases, it was possible to detect and quantify ecdysterone around 1–2 h after the intake, indicating that ecdysterone is rapidly eliminated from the blood to the urine.

The maximum excretion rate of ecdysterone after sautéed spinach was found in the range of 2.6–27.2 µg h^{-1} in the 3–6 h samples (midpoint time). In the case of smoothie ingestion, the maximum excretion rate was 7.6–27 µg h^{-1} and reached between 1.5 and 7 h midpoint time. The cumulative amount of ecdysterone was 25.4–163.4 µg following sautéed spinach intake, and 52–272 µg after smoothie intake.

All participants showed a similar pattern for the excretion rate of ecdysterone, however, in one of the participants it was much lower compared to the others. It seems that the subject has a poor ability in the absorption of ecdysterone (also confirmed in the second administration). In most of the subjects after 10–15 h, the cumulative amount of ecdysterone reached a plateau, indicating that the excreted ecdysterone in the following time is not significant anymore. After 24 h ecdysterone was no longer detectable (c < LOD). In line with earlier reports, the half-life of ecdysterone was found short. Based on the excretion rate method, the half-life of ecdysterone after the intake of sautéed spinach and smoothie is between 2 and 4 h. The short half-life and concomitant fast excretion may explain why ecdysterone cannot be detected anymore after 24 h.

The first metabolite of ecdysterone, 14-deoxy-ecdysterone, was excreted and quantified in all eight participants following sautéed spinach intake. On the other hand, only in six out of eight

Table 2. Summary of urinary elimination data of ecdysterone and its metabolites 14-deoxy-ecdysterone and 14-deoxy-poststerone after ingestion of sautéed spinach or smoothie.

	Sautéed spinach				Smoothie			
	n	Range	Mean (SD)	Median (IQR)	*n*	Range	Mean (SD)	Median (IQR)
Ecdysterone	8				8			
Cmax [μg mL^{-1}]		0.09–0.41	0.22 (0.13)	0.16 (0.23)		0.08–0.74	0.23 (0.22)	0.13 (0.19)
Erate-max [μg h^{-1}]		2.6–27.2	17.02 (7.03)	17.6 (5.15)		7.6–26.8	18.8 (6.3)	20.35 (8.66)
Cumulative amount [μg]		25.4–163.4	127.90 (44.32)	144.07 (33.39)		52.8–272.3	137.80 (66.5)	122 (68.4)
Recovered in urine [%]		0.14–0.86	0.66 (0.23)	0.73 (0.17)		0.28–1.82	0.79 (0.47)	0.66 (0.4)
14-deoxy-ecdysterone	8				6			
Cmax [μg mL^{-1}]		0.03–0.67	0.17 (0.24)	0.04 (0.21)		0.02–0.26	0.09 (0.09)	0.06 (0.09)
Erate-max [μg h^{-1}]		1.02–51.1	9.9 (17)	3.13 (7.30)		1.45–12.7	7.5 (5.3)	8 (9.95)
Cumulative amount [μg]		10–406	83.2 (138.4)	19.63 (83.40)		7.4–199	78 (81)	43.5 (138)
Recovered in urine [%]		0.05 – 2.01	0.42 (0.69)	0.10 (0.44)		0.04 – 1.08	0.45 (0.48)	0.23 (0.95)
14-deoxy-poststerone	3				3			
Cmax [μg mL^{-1}]		0.04–0.4	0.18 (0.19)	0.10 (0.35)		0.04–0.36	0.15 (0.18)	0.06 (0.32)
Erate-max [μg h^{-1}]		3.3–13	6.7 (5.5)	3.72 (9.76)		1.9–23.4	10.4 (11.5)	5.8 (21.52)
Cumulative amount [μg]		47–380	159 (191)	51 (333)		38–303	137 (144)	70.4 (265)
Recovered in urine [%]		0.25–2	0.84 (1.0)	0.27 (1.74)		0.25–1.69	0.77 (0.8)	0.38 (1.44)

n, number of participants who excreted the compound in urine.

participants the metabolite could be quantified after smoothie administration. In the other two participants, 14-deoxy-ecdysterone was detected but not quantified as the concentration was below the lowest calibration point, yet the signal to noise of these samples is higher than 3 (i.e., LOD).

In the case of 14-deoxy-ecdysterone after sautéed spinach administration, maximum concentrations of 0.03–0.67 μg mL^{-1} were obtained between 9 and 21 h. Similarly, in the smoothie administration, the maximum concentration was obtained in the range 0.02–0.26 μg mL^{-1} at urine sampling times between 9 and 30 h. The maximum excretion rate was in the range 1–51 μg h^{-1} and obtained between 5 and 22 h midpoint sampling time for sautéed spinach. In the second administration, it was in 1.5–12.7 μg h^{-1} range and observed at 6–25 h midpoint time. The cumulative excreted amount of 14-deoxy-ecdysterone was 10–406 and 7.4–199 μg following sautéed spinach and smoothie, respectively.

The concentrations and the excretion rates of 14-deoxy-ecdysterone showed high variability between the subjects. This metabolite was detected until 24 h in 75% of subjects and up to almost 2 days in the last remaining subjects. The cumulative amount of 14-deoxy-ecdysterone reached a plateau at different times which also indicated its high interindividual variability. Some subjects reached the plateau already in 10–15 h while for the rest at this time the cumulative amount is still increasing, and the plateau was reached only after 30 h.

According to Kumpun et al.,[24] 14-deoxy-ecdysterone is most likely generated by gut microbiota (at least in mice). Thus, the variation of 14-deoxy-ecdysterone in this study might be a result of the involvement of gut microbiota as it is highly influenced by genetic, diet, the use of antibiotic, health condition, and environmental conditions of each person.[34]

In addition to ecdysterone and 14-deoxy-ecdysterone, another metabolite, i.e., 14-deoxy-poststerone, was excreted in the urine, however only detectable in three out of eight participants. The three participants who excreted this metabolite after the intake of sautéed spinach were the same participants that also excreted the metabolite after smoothie intake as can be seen in Figure 3. All three participants were female. Taken together with findings from earlier studies,[27] the metabolite 14-deoxypoststerone was only detected in female volunteers until now, which opens new perspectives for further investigations of potential sex related differences in metabolism in the future.

In 14-deoxy-poststerone, the maximum concentration after both interventions was in a similar range (0.04–0.4 μg mL^{-1} for sautéed spinach and 0.04–0.36 μg mL^{-1} for smoothie ingestion) and observed between 18 and 25 h post-administration in both administrations. The maximum excretion rate was determined with 3.3–13 μg h^{-1} at 13–21 h for sautéed spinach and between 1.9 and 23.4 μg h^{-1} at 18–23 h (midpoint sampling time) for smoothie intake. Based on the graph shown in Figure 3, the same subject showed the highest concentration and excretion rate for 14-deoxy-poststerone compared to the other two subjects both, in sautéed spinach and smoothie intake. The cumulative amount of 14-deoxy-poststerone in urine was 47–380 and 38–303 μg for sautéed spinach and smoothie consumption, respectively, and reached its plateau around 20–30 h in both administrations.

The summary of urinary excretion parameters for sautéed spinach and smoothie ingestion is presented in **Table 2**.

3.3. Recovered Amount (%) of Ecdysterone and Its Metabolites in Urine Compared to Pure Ecdysterone Intake

Based on the analytical data and excreted volume of urine, the mean (SD) recovered amount (%) of ecdysterone, 14-deoxy-ecdysterone and 14-deoxy-poststerone was calculated as 0.66 (0.23), 0.42 (0.69), 0.84 (1.0) for sautéed spinach and

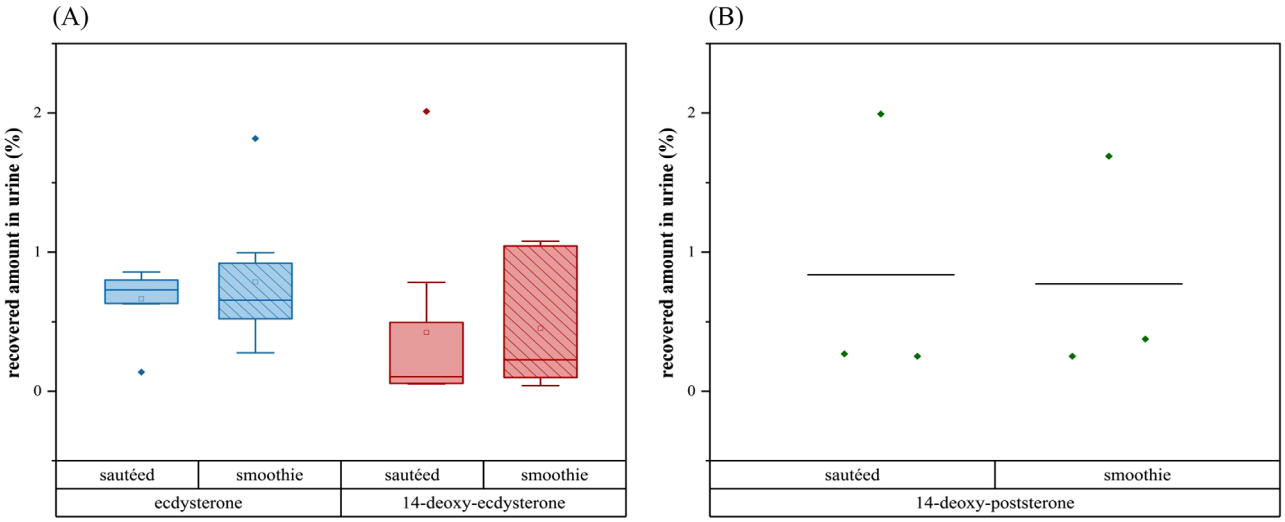

Figure 4. Box-plots of recovered amount in urine (%) for ecdysterone and 14-deoxy-ecdysterone (A), and individual point of recovered amount in urine (%) for 14-deoxy-poststerone (B) after sautéed spinach and smoothie administration.

0.79 (0.47), 0.45 (0.48), 0.77 (0.8) for smoothie, respectively (**Figure 4**). Only small percentages of the ingested ecdysterone are recovered in urine after the two different types of spinach administration: 0.14–0.86% after the sautéed spinach and 0.28–1.82% after smoothie. This might indicate that ecdysterone is poorly absorbed from spinach. Additionally, breaking the leave structure in the blender does not significantly improve the availability of ecdysterone. While the same metabolites were detected in urine samples collected after the administration of pure ecdysterone, significant differences were observed in terms of quantities. After oral administration of pure ecdysterone 18 ± 13% of the administered ecdysterone was recovered in the urine.[26] Comparing these data show that the amount of ecdysterone that was recovered after spinach intake was in the range 1/27–1/23 of the amount recovered following the intake of pure ecdysterone. If no limited process in the absorption phase takes place, the recovered amount (%) from pure ecdysterone administration and spinach intake should be equal or at least similar. This gives a hint that spinach as food is indeed a poor source of ecdysterone. One possible explanation may be that ecdysterone itself is trapped inside the cells and thus cannot be absorbed. Alternatively, the volume of spinach food (in this study almost 1 kg) may decrease the solubility of ecdysterone in the gastrointestinal fluids which might reduce the maximum absorption capacity of ecdysterone.

Thus, it would be interesting to investigate the mechanism of absorption of ecdysterone from spinach. Ecdysterone should first be released from the food matrix to become bioaccessible at the site of absorption, then dissolved, as only the released form can be absorbed. Preliminary data suggest that ecdysterone absorption from spinach is limited to a certain amount, which further influences the concentration of ecdysterone excreted into the urine. An administration of different amounts of ecdysterone from spinach is suggested for further elucidation.

Similar to the recovery of ecdysterone parent, also only small percentages of the ingested ecdysterone were recovered in urine as 14-deoxy-ecdysterone and 14-deoxy-poststerone. In case of the metabolite 14-deoxy-ecdysterone recovery in urine (as percentage in comparison to the ingested ecdysterone) ranged 0.05–2% after sautéed spinach ingestion and 0.04–1.08% after smoothie. In the relevant subjects ($n = 3$, see Section 3.2), the metabolite 14-deoxy-poststerone was recovered in urine in the range of 0.25–2% for sautéed spinach and 0.25–1.7% for smoothie.

Non-parametric Wilcoxon signed-rank test was performed for comparing the maximum concentration and recovered amount (%) in urine for ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone between sautéed spinach and smoothie. No significant differences were observed for ecdysterone and both metabolites in terms of maximum concentration and recovered amount (%) between the two intakes.

The boxplot of the total excreted amount (%, as sum of ecdysterone and its metabolites) between sautéed spinach and smoothie (A) and the individual recovered amount (%) in urine for both intakes (B) is displayed in **Figure 5**. While after both sautéed spinach and smoothie intake the mean (SD) of the total excreted amount (%) in the urine was only 1.4 (1.0), after oral intake of pure ecdysterone it was 21.1 (13.3), as the total amount of unchanged drug and metabolites in our previous study.[27] The recovered amount was 15 times higher from oral solution of pure ecdysterone than from spinach intake. This information also implies that ecdysterone is poorly absorbed when the source is spinach.

The paired-sample *t*-test showed no significant difference for the total excreted amount between the two intakes. The intraindividual recovered amount (%) showed a quite similar proportion of excreted ecdysterone and its metabolites between two intakes. On the other hand, there was interindividual variation between subjects for the extent of the excreted amount of ecdysterone and its metabolites in urine (Figure 5). Ecdysterone was the most abundant compound excreted in six out of eight participants. In the other two participants, one participant excreted 14-deoxy-ecdysterone as the most dominant compound, while 14-deoxy-poststerone was the major excreted compound in another participant.

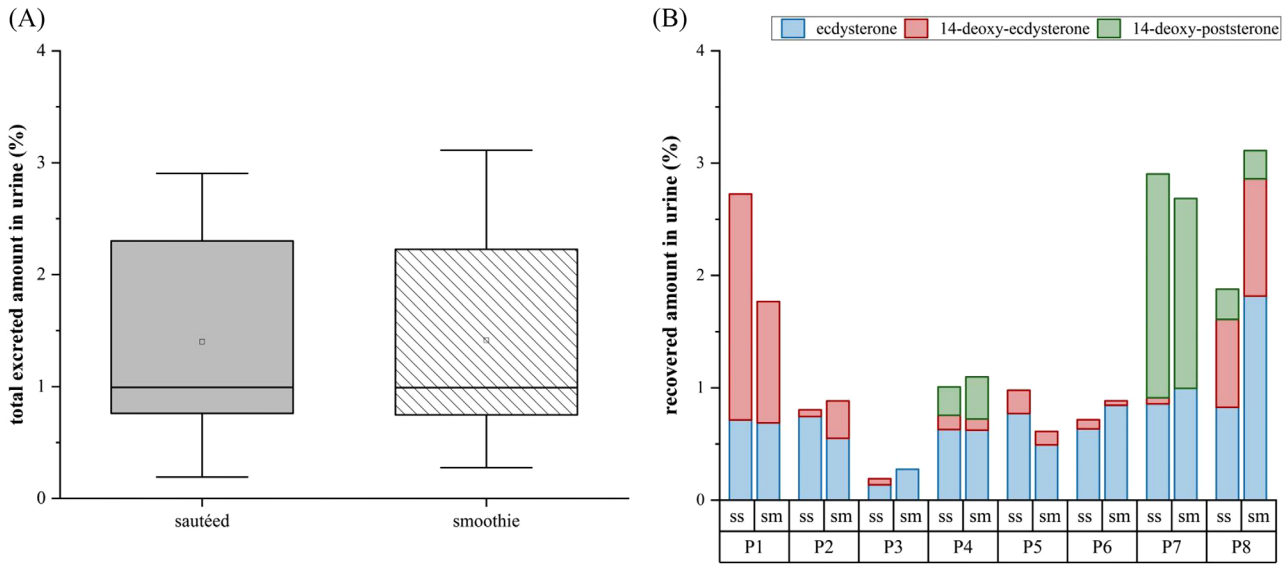

Figure 5. The box-plots of total excreted amount (%) in urine (A) and the individual recovered amount (%) in urine (B) after sautéed spinach (ss) and smoothie (sm) intake.

4. Conclusion

Only a small proportion of ecdysterone from spinach was excreted into urine. No significant differences were found in concentration and recovered amount (%) of ecdysterone, 14-deoxy-ecdysterone, and 14-deoxy-poststerone in urine between sautéed spinach and smoothie ingestion. Thus, very high amounts of spinach consumption may be needed to achieve health related effects from ecdysterone ingestion via spinach. Further studies on the potentially beneficial high-spinach diet to improve conditions correlated with muscle wasting, such as sarcopenia etc., will be addressed in future studies. In sports doping control, a discrimination between ecdysterone from food or preparations, will be challenging based on urinary concentrations only at least for later post-administration samples. A threshold concentration may be set to identify samples recently collected after excessive misuse, however later elimination phases may not be covered. Further investigations on multiple-dose administrations and further potential biomarkers will be conducted in the near future.

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

Conceptualization, M.K.P.; methodology, T.Y., K.T., S.L., S.V., and E.I.; formal analysis, T.Y. and K.T.; investigation, T.Y. and K.T.; resources, P.D., F.B., and M.K.P.; writingoriginal draft preparation, T.Y. and K.T.; writingreview and editing, M.K.P., F.B., and X.T.; visualization, T.Y. and K.T.; supervision, M.K.P. and F.B.; project administration, M.K.P.; funding acquisition, M.K.P., F.B., P.D., and X.T. All authors agree with submission and publication.

Data Availability Statement

Raw data are stored by the authors.

Keywords

doping, ecdysterone, kinetics, metabolites, phytosteroid, spinach, urine

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