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Development and Validation of High-Performance Liquid Chromatography for Identification and Quantification of Phytoecdysteroids Ecdysterone and Turkesterone in Dietary Supplements

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Abstract: Phytoecdysteroids are a group of naturally occurring ecdysteroid hormones found in certain plant families and used for centuries for their adaptogenic, tonifying, and antioxidant properties. Ecdysterone and turkesterone are the dominant phytoecdysteroids found in the plant species *Rhaponticum carthamoides* Willd., *Cyanotis arachnoidea* C.B.Clarke and *Ajuga turkestanica* (Regel) Briq., and have been recognized for their adaptogenic potential and ability to enhance physical performance. However, over the past few years, supplementation with ecdysterone by professional athletes has raised some concerns about its safety and quality, leading to its inclusion in the World Anti-Doping Agency monitoring program. This study proposes a simple and reliable method for quality control of ecdysterone- and turkesterone-containing products using high-performance liquid chromatography (HPLC) analysis. The HPLC method is based on reversed-phase chromatography and gradient elution to achieve a superior separation of phytoecdysteroids. The method has been shown to qualify both phytoecdysteroids at low concentrations such as 10.98 µg/mL for ecdysterone and 11.43 µg/mL for turkesterone. This method could be successfully used in research programs and routine quality control of dietary supplements to ensure their safety in professional athletes' nutrition.

Keywords: phytoecdysteroids; 20-hydroxyecdysterone; turkesterone; high-performance liquid chromatography; dietary supplements; *Rhaponticum carthamoides* Willd

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1. Introduction

Ecdysteroids (EDs) are a group of steroid hormones that occur naturally in plants and insects [1,2]. Nowadays, more than 550 EDs have been identified and classified on the basis of their natural origin as phyto-, zoo-, and myco-ecdysteroids [2,3]. Certain plant families such as Asteraceae, Liliaceae, Lamiaceae, Magnoliaceae, Basellaceae, Podocarpaceae, Commelinaceae, Ranunculaceae, etc. contain phytoecdysteroids (PEs), including species such as *Rhaponticum carthamoides* Willd. *(R. carthamoides)*, *Podocarpus nakaii*, *Serratula coronate*, *Ajuga turkestanica* (Regel) Briq. *(A. turkestanica)*, *Cyanotis arachnoidea* C.B.Clarke *(C. arachnoidea)*, etc. [2,4,5].

These plants have been used since ancient times for their adaptogenic and tonifying effects, to promote muscle growth, and for their antioxidant properties [6–9]. Among the complex of PEs found in these plants are 20-hydroxyecdysterone (ecdysterone), turkesterone, ponasterones A, B, and C, ajugasterone C, polypodine B, etc. [4,10]. The dominant PEs in *R. carthamoides*, *A. turkestanica*, and *C. arachnoidea* are 20-hydroxyecdysterone and turkesterone [6,11,12]. Guibolt et al. reported that *A. turkestanica* contains approximately 70% of all isolated PEs as turkesterone, while the amount of ecdysterone is about 10 times less [11]. In *R. carthamoides*, the content of 20-hydroxyecdysterone is in the range of 0.04–1.51% of the plant dry matter [6].These PEs—ecdysterone and turkesterone—have been associated with several biological effects, such

as anabolic, neuroprotective, antidiabetic, and antihyperlipidemic [13–16]. Ecdysteroids have been recognized for their pharmacological properties, particularly their ability to enhance physical performance through beneficial changes in body composition and anabolic effects, mediated by the activation of β-estrogen receptors [17]. In this regard, ecdysterone, as a typical ecdysteroid, has been found to affect anabolic pathways through estrogenic receptor pathways, without androgenic side effects, making it an attractive supplement for athletes [18,19] The structure-activity relationship of PDs remains incompletely understood. These compounds are a class of polyhydroxylated sterols structurally similar to androgens [2,14,20]. They contain a carbon skeleton—cyclopentanoperhydrophenanthrene with a β-side chain at C17 [20]. Notably, some hydroxyl groups are believed to play an important role in the biological activity of PDs. The hydroxyl groups at the C-2, C-3, C-14, C-20, and C-22 positions as well as the double bond at C-7 and the keto group at C-6 are suggested to be particularly important for their biological activity. The anabolic activity of PDs is associated with the presence of a hydroxyl group at C-20, while the presence of a hydroxyl group at C-11 is essential for the anabolic activity of turkesterone (Figure 1) [21–24].

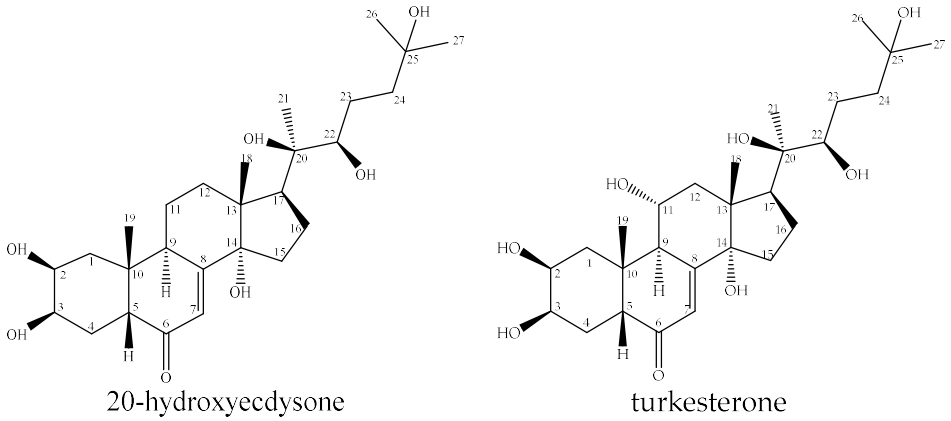

Figure 1. Chemical structures of ecdysterone and turkesterone.

Since 2020, ecdysterone has been included in the World Anti-Doping Agency (WADA) monitoring program as an anabolic agent (in both competitive and non-competitive environments) due to its ability to enhance athletic performance [25,26]. Moreover, 20-hydroxyecdysterone is usually marked as "natural anabolic agent" [27]. Although many researchers suggest that ecdysterone supplementation has been used by Soviet athletes since the 1980s and it was sometimes called the "Russian secret" (*R. carthamoides* naturally grows in Russia), nowadays supplementation with *R. carthamoides* and other products containing ecdysterone is common practice among athletes from all over the world, because of the high adaptogenic potential of the molecule [8,18,27–29]. The intake of *R. carthamoides* extracts and its contained secondary metabolites, ecdysterone, and turkesterone, has been associated with multiple athletic performance benefits [30–33], as shown in Figure 2.

Dietary supplements (DSs) play a crucial role in professional athletes' regime and preparation. Most of these products are carefully selected by the athlete's team, and their intake aims not only to improve the athletic performance but also the immune system of athletes to shorten the time needed for recovery [34]. However, unlike medicines, DSs are not tested for quality before being released on the market, and manufacturers are not required to provide information on their efficacy or safety before introducing them [35]. This liberalization of regulations regarding DSs has led to concerns about their quality and safety [35]. Over the past two decades, there have been many cases of great differences between the label of some DSs and their actual composition. Therefore, the development of new, rapid, precise, accurate and sustainable methods for the analysis of DSs containing EDs with appropriate techniques is required [18]. Among the various techniques available, high-performance liquid chromatography (HPLC) is one of the most widely used techniques

to ensure the quality control of DSs. It is characterized by its capabilities in terms of separation and quantification of the components, due to its high resolution, sensitivity and versatility [36–39]. The aim of the current study is to propose, develop, and validate a simple, fast, and reliable approach for identifying 20-hydroxyecdysterone and turkesterone in dietary supplements using HPLC analysis.

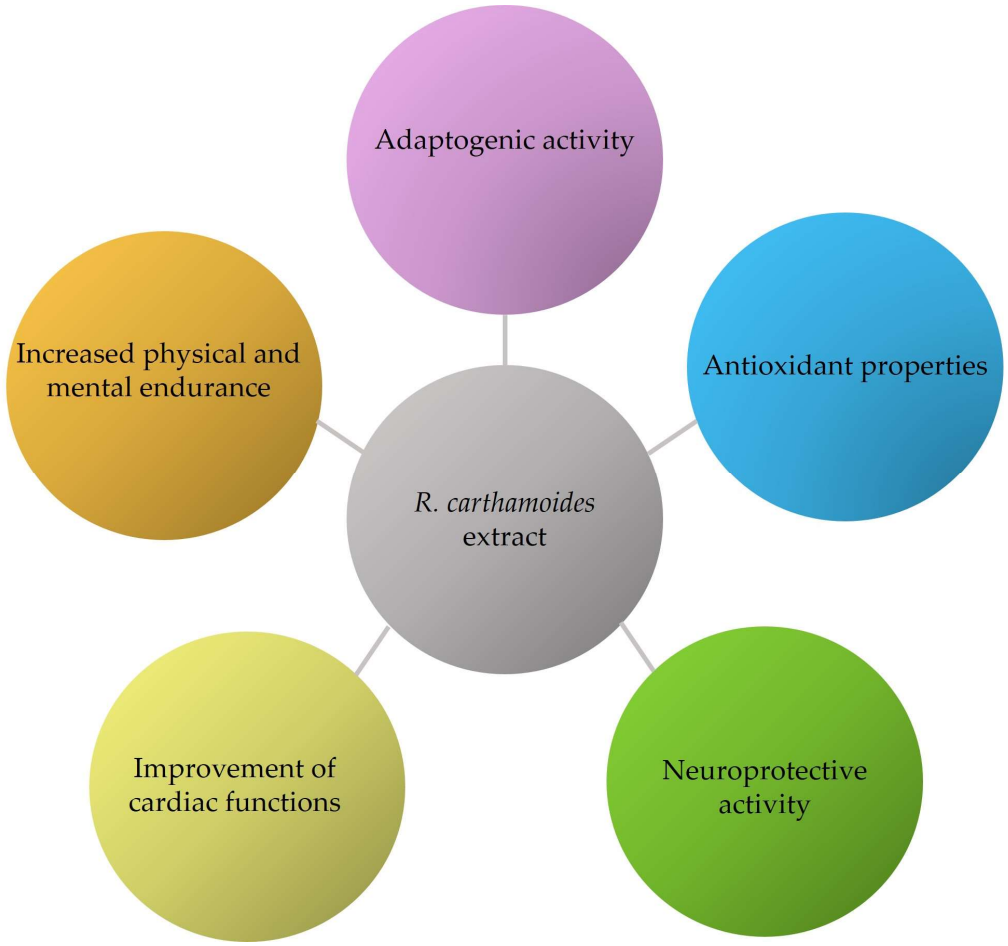

Figure 2. Effects of *R. carthamoides* extracts.

2. Materials and Methods

2.1. Chemicals

The reference standards of 20-hydroxyecdysterone, HPLC ≥ 95%, and turkesterone, HPLC ≥ 95% were obtained from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). HPLC grade acetonitrile was obtained from Merck KGaA (Darmstadt, Germany). Prior to usage, the mobile phase solvents were subjected to filtration through 0.45 µm membrane filters (Millipore, Milford, MA, USA) and degassed using sonic waves.

2.2. Samples

Eleven dietary supplements used in the study were randomly selected and purchased online.

2.3. HPLC Analyses

2.3.1. Standard Solutions

Stock solutions for HPLC analysis of 20-hydroxyecdysterone and turkesterone were prepared in acetonitrile at a concentration 1 mg/mL, and an ultrasonic bath (Bandelin,

Berlin, Germany) was used for better dissolution. The prepared stock solutions were stored in brown vials, protected from light, at 4 °C until use.

2.3.2. Sample Solutions

For the purpose of analysis, tablet, and capsule samples were ground and weighted before dissolving. They were extracted with 10 mL acetonitrile and sonicated for 15 min. Then, they were filtered through a 0.45 µm syringe filter and subsequently diluted before injection. Samples were prepared in triplicate.

2.3.3. Instrumentation

HPLC analyses were performed on a Varian Pro Star HPLC system (Mulgrave, Victoria, Austria) with a variable wavelength UV-VIS detector. Analysis was conducted on an Agilent -C18 column (5 mm × 250 mm, 4.6 µm), thermostated at 45 °C. The injection volume was 20 µL and a Hamilton syringe was used for manual injection. UV detection was set at λ = 242 nm.

2.3.4. Method Development

Gradient conditions of the mobile phase were employed to achieve better separation of the PDs. Initially, the mobile phase consisted of acetonitrile and water (95:5, v/v) at a flow rate of 0.8 mL/min. The flow rate was increased to 1 mL/min in the second minute, while in the third minute the acetonitrile: water ratio was changed to 98:2 (v/v). After the eighth minute, the mobile phase was returned to its initial composition.

2.3.5. Method Validation

The method was validated according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) with the following validation parameters: linearity range, accuracy, precision, limit of detection and quantification, and robustness [40].

3. Results

3.1. Method Development

In order to achieve a better PD separation, gradient conditions were employed for the mobile phase. Initially, the mobile phase consisted of acetonitrile and water (95:5, v/v) at a flow rate of 0.8 mL/min. In the second minute, the flow rate was increased to 1 mL/min, and in the third minute the acetonitrile: water ratio was changed to 98:2 (v/v). After the eighth minute, the conditions were returned to the initial ones with a ratio of acetonitrile: water (95:5, v/v) at a flow rate of 0.8 mL/min. The gradient mobile phase yielded a good peak shape and symmetry. Moreover, the selectivity factor (α) between the compounds examined was 1.8, indicating good peak separation and differentiation. The resolution (Rs) between these peaks was determined to be 2.4. These parameters, the selectivity factor and resolution, demonstrate the efficiency and effectiveness of the chromatographic separation method. The retention times of the analytes were as follows: 3.902 min for 20-hydroxyecdysterone and 4.554 min for turkesterone. Figure 3 shows the HPLC chromatograms of the analyzed phytoecdysteroids.

After employing gradient solvent system conditions and a reversed-phase stationary phase, the developed HPLC method allowed the separation of the compounds in only six minutes. Due to the rapidity and simplicity of the method, it was subsequently validated.

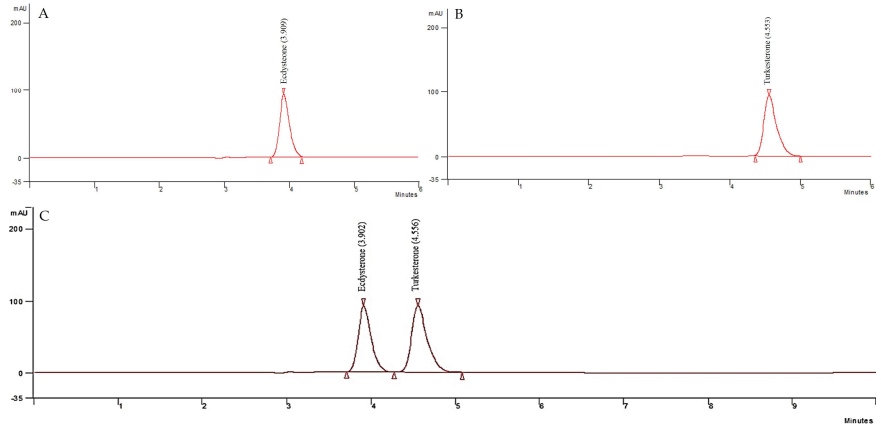

Figure 3. (**A**) HPLC chromatogram of the standard solution of 20-hydroxyecdysone (25 µg/mL) with a retention time of 3.902 min. (**B**) HPLC chromatogram of the standard solution of turkesterone (25 µg/mL) with a retention time of 4.554 min. (**C**) HPLC chromatogram of the standard solutions of 20-hydroxyecdysterone and turkesterone.

3.2. Method Validation

The method validation was conducted according to ICH guidelines [40].

Linearity

Linearity refers to the capacity of an analytical procedure to generate assay results proportional to the analytic concentration in the sample range. At least five concentrations are required to establish linearity, and this was accomplished using an external standard curve. The linearity of the developed method was assessed by measuring five concentrations and their corresponding peak areas. The observed range of linearity was from 12.5 to 75 µg/mL. The linear regression line for ecdysterone was y = 146,533x − 49,820, with a high correlation coefficient R^2 = 0.9997, while the linear regression line for turkesterone was y = 142,462x − 4359.1, with R^2 = 0.9996; the calibration curves are shown in Figure 4.

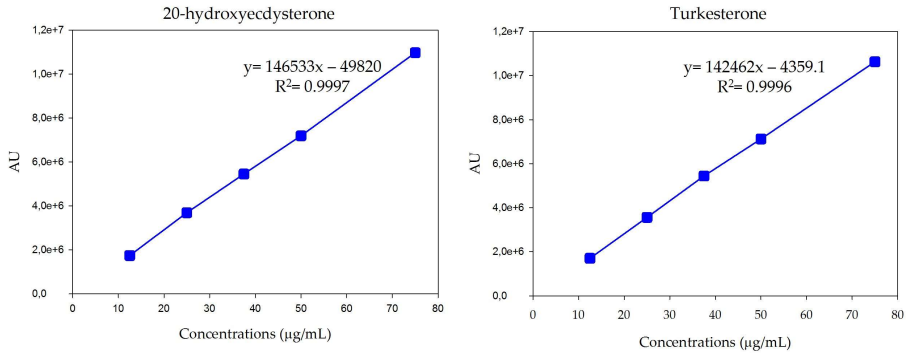

Figure 4. Calibration curves of 20-hydroxyecdysterone and turkesterone.

Accuracy and precision

Accuracy, also referred to as trueness, was verified throughout the designated range of the analytical method. To verify the accuracy of the developed method, a recovery test was conducted at three known concentration levels. The concentration levels used were as follows for ecdysterone (high—50 µg/mL, medium—25 µg/mL, and low—16 µg/mL qualitative concentrations), and turkesterone (high—50 µg/mL, medium—25 µg/mL, and low—16 µg/mL qualitative concentrations). Accuracy was reported as a percentage recovery calculated as the difference between the mean and assumed true values. The percentage recovery of 20-hydroxyecdysterone was in the range of 99.99–100.89%, and

that of turkesterone was 99.96–100.09%. This demonstrates the acceptable accuracy of the method. Table 1 shows the results of the accuracy assessment for the tested PDs.

Table 1. Results on the accuracy of the developed HPLC method for the investigated phytoecdysteroids.

Concentration (μg/mL)	Mean (μg/mL) ± SD	Recovery %	CV%
20-hydroxyecdysterone			
50	49.93 ± 0.18	99.85	0.36
25	25.06 ± 0.14	100.25	0.55
16	16.10 ± 0.08	100.65	0.51
Turkesterone			
50	50.16 ± 0.27	100.32	0.54
25	24.93 ± 0.11	99.73	0.44
16	16.02 ± 0.19	100.11	1.19

CV%—Coefficient of variation.

Precision in analytical procedures refers to the degree of closeness or agreement between multiple measurements of the same homogeneous sample obtained under the same conditions. The precision of the method was determined as inter-day and intra-day precision, by evaluating the coefficient of variation for the three known level concentrations, each concentration with six replicates. Table 2 presents the results of the precision of the developed method.

Table 2. Precision evaluation of the developed HPLC method.

Concentration (μg/mL)	Intra-Day Precision			Inter-Day Precision		
	Mean (μg/mL) ± SD	Standard Error	CV%	Mean (μg/mL) ± SD	Standard Error	CV%
20-hydroxyecdysterone						
50	49.94 ± 0.18	0.07	0.35	49.99 ± 0.23	0.09	0.47
25	25.08 ± 0.15	0.06	0.61	25.02 ± 0.08	0.03	0.33
16	16.08 ± 0.07	0.03	0.41	15.99 ± 0.05	0.02	0.30
Turkesterone						
50	50.03 ± 0.08	0.03	0.15	50.03 ± 0.08	0.03	0.15
25	25.02 ± 0.07	0.03	0.26	25.04 ± 0.05	0.02	0.19
16	15.99 ± 0.07	0.03	0.42	16.01 ± 0.09	0.04	0.59

The values of correlation coefficients close to unity, the high percentage of accuracy, and the low values of standard deviation indicate that the developed method is linear, accurate, precise, and reliable for the determination of 20-hydroxyecdysterone and turkesterone.

Limit of detection (LD) and limit of quantification (LQ)

The detection limit refers to the minimum level of analyte that can be detected in a sample, while the quantification limit refers to the minimum level of analyte that can be accurately and precisely determined in a sample. These were calculated from the linearity data using the standard deviation of a linear response and a slope, and were found to be 3.62 μg/mL and 10.98 μg/mL for 20-hydroxyecdysterone, respectively. While for turkesterone, LD and LQ were evaluated as 3.77 μg/mL and 11.43 μg/mL, respectively.

Robustness

Robustness indicates the reliability of an analytical procedure with respect to deliberate variations in chromatographic parameters. It was evaluated by determined variations in column temperature. To assess the effect of temperature variations, specific temperatures of 42–48 °C were deliberately chosen and the retention time of each compound was evaluated. The resolution did not undergo significant changes when the temperature was modified. Method robustness shows that minor changes in the temperature do not lead to changes in the chromatographic separation.

Stability

Neither ecdysterone nor turkesterone are thermolabile compounds. The standard solutions were stored in a refrigerator (2–8 °C). Clarity of the solutions was examined visually and they were analyzed using the developed HPLC method. By comparing the chromatograms obtained from the freshly prepared solutions with those from the stored ones, it was determined that the samples remained stable throughout the entire storage period. This indicates that the samples can be reliably stored and analyzed using the developed HPLC method without significant degradation or changes in their chemical properties.

Previously reported studies on the analysis of PDs in different plant extracts have used various HPLC methods. Poojari et al. used an HPLC method with a reversed-phase stationary phase, a column thermostat at 25 °C, and a mobile phase consisting of methanol: water or 0.01 M KH_2PO_4: methanol in a gradient elution method to analyze ecdysterone in *Sida rhombifolia subsp.retusa* and *Embelia tsjeriam-cottam* [41]. Liktor-Busa et al. reported on both normal-phase and reversed-phase HPLC methods, using various mobile phases for chromatographic separation of EDs in *Serratula wolffii*. The mobile phases used for normal-phase chromatography were cyclohexane: isopropanol: water (100:40:3, *v*/*v*/*v*); dichloromethane: isopropanol: water (125:40:3, *v*/*v*/*v*); dichloromethane: isopropanol: water (125:30:2, *v*/*v*/*v*); and dichloromethane: isopropanol: water (125:25:2, *v*/*v*/*v*). While in reversed-phase HPLC, the mobile phases used were acetonitrile: water (35:65, *v*/*v*); acetonitrile: water containing trifluoroacetic acid (0.1%) (77:23, *v*/*v*); and methanol: water (6:4, *v*/*v*) [42]. Dinan et al. and Głazowska et al. have also systematized previously reported HPLC methods for EDs identification, which include both reversed-phase and normal-phase chromatography with various mobile phase conditions utilizing mixtures of methanol: water or acetonitrile: water and a low-polarity organic solvent, such as dichloromethane, chloroform, or ether, and a lower proportion of the polar organic component, such as methanol, ethanol, or isopropanol, for the normal phase [43,44].

The method developed and validated in this study can be easily reproduced and it is also characterized by easy sample preparation, good linearity, accuracy, precision, and reliability. In comparison to previous methods for the analysis of ecdysteroids in plant extracts, the use of a reversed-phase stationary phase is suitable for the separation of ecdysteroids, while for the mobile phase, acetonitrile: water with gradient elution is more appropriate. Moreover, in the present method, pH is not adjusted to acidity, in contrast to some previously established methodologies. As a result, this approach is considered to be more environmentally friendly. Indeed, the highly acidic mobile phase can lead to degradation of HPLC column packing material, thereby causing harmful effects such as loss of resolution and peak distortion. Furthermore, acidic mobile phases can damage some components of the HPLC system. The developed HPLC technique is user friendly and time saving, because this method eliminates the need for prior derivatization of the phytoecdysteroid in contrast to gas chromatography [27] and avoids the complex and tangled sample preparation which is required for HPLC-MS techniques [28,45].

3.3. Method Application—Analysis of Dietary Supplements

A study conducted by Lafont et al. revealed that over 140 different preparations containing EDs are commercially available for oral consumption, which can be categorized into two categories: those containing crude or semi-purified plant extracts, such as plant powders or alcoholic extracts, and those containing "pure" 20-hydroxyecdysterone or a defined ecdysteroid mixture. Most of these preparations are marketed to bodybuilders. The significant growth

of ecdysteroid-based products on the market suggests that these molecules possess at least some of the claimed effects [46]. The increasing number of products containing ecdysteroids leads to the need for implementation of appropriate and reliable methods for their analysis. The developed and validated rapid HPLC method was applied to identify and qualify ecdysterone and turkesterone in eleven randomly selected DSs labeled as plant extracts of *C. arachnoidea*, *R.carthamoides* and *A.turkenistanica*. The estimated concentrations of ecdysterone in the analyzed dietary supplements are presented in Table 3.

Table 3. Concentrations of 20-hydroxyecdysterone and turkesterone in dietary supplements, where ES = ecdysterone and TS = turkesterone.

Sample №	Country of Origin of the Manufacturer	Sample Type	Product Description	ES Content (mg/g)	Determined ES Content (mg/Capsule or Tablet)	TS Content (mg/g)	Determined TS Content (mg/Capsule or Tablet)	ES/TS Labeled Content	Labeled Extract Content (mg/Capsule or Tablet)
1	Czech Republic	Capsule	*C. arachnoidea* extract	188.07 mg/g	87.00 mg	38.33 mg/g	17.73 mg	90% ecdysterone	300 mg
2	American brand; where produced not labeled	Capsule	*R. carthamoides* extract	302.24 mg/g	91.88 mg	1.06 mg/g	0.32 mg	Not labeled	250 mg
3	Bulgaria	Capsule	β-ecdysterone from *R. carthamoides* extract	44.16 mg/g	19.30 mg	2 mg/g	0.80 mg	95% ecdysterone	245 mg
4	American brand; where produced not labeled	Capsule	*R. carthamoides* extract	232.71 mg/g	79.82 mg	57.73 mg/g	19.80 mg	80% ecdysterone	250 mg
5	Bulgaria	Tablet	*R. carthamoides* extract	17.52 mg/g	5.29 mg	0.75 mg/g	2.48 mg	Not labeled	200 mg
6	Bulgaria	Tablet	*R. carthamoides* extract	151.17 mg/g	14.80 mg	12.97 mg/g	1.27 mg	15 mg	35 mg
7	Bulgaria	Tablet	*R. carthamoides* extract	125.06 mg/g	16.32 mg	0.92 mg/g	0.12 mg	16.5 mg	40 mg
8	Czech Republic	Capsule	*R. carthamoides* extract	61.65 mg/g	35. 14 mg	-	-	300 mg	330 mg
9	Sweden	Capsule	*A. turkestanica* extract + Ginseng and Astragal extract	0.87 mg/g	0.56 mg	80.14 mg/g	51.77 mg	Min. 10% turkesterone	500 mg
10	Bulgaria	Capsule	*A. turkestanica* extract	178.27 mg/g	47.26 mg	1.06 mg/g	0.64 mg	10 % turkesterone	500 mg
11	American brand; where produced not labeled	Capsule	*A. turkestanica* extract	40.35 mg/g	19.64 mg	12.80 mg/g	6.23 mg	10 % turkesterone	500 mg

In the present investigation, a total of 11 DS samples containing different types of plant extracts were tested. One of the samples contained *C. arachnoidea* extract, seven contained *R. carthamoides* extract, and three contained *A. turkestanica* extract. Considering the presence of ecdysterone and turkesterone in the extracts of *C. arachnoidae*, *R. carthamoides*, and *A. turkestanica*, both PDs were not only detected but also quantified in all of the tested DSs. Two of the samples that were reported to contain *R. carthamoides* extract, had an exact amount of ecdysterone of 15 mg and 16.5 mg, respectively, as confirmed by our analysis. These DSs are available not only in online stores but also in pharmacies and drugstores.

We have found that in some samples the content of ecdysterone differs significantly from the labeled. For example, sample No. 8 was labeled as *R. carthamoides* extract 330 mg, which contained 300 mg ecdysterone. The label of this product is definitely misleading—330 mg extract cannot contain 300 mg ecdysterone. The actual content of ecdysterone in this sample was 35.14 mg/capsule. It is also disturbing and quite unusual that turkesterone was not detected in this sample, which suggests a poor quality extract. Moreover, samples Nos. 1, 3, and 4 were labeled to contain *R. carthamoides* extract 300 mg, 245 mg, and 250 mg, respectively, and also labeled to contain 90% ecdysterone, 95% ecdysterone, and 80% ecdysterone, respectively. In these DSs the content of ecdysterone differed significantly from what is labeled. In these cases the qualified ecdysterone is much less than the labeled one. However, in these samples, turkesterone was detected. Previously, Ambrosio et.al. and Kraiem et. al. have reported such parallel results—poor labeling of similar DSs. These researchers reported that the DSs samples they examined contained quite different concentrations of ecdysterone than the labels indicated [17,45].

Regarding dietary supplements containing *A. turkestanica*, only sample No. 9 contained approximately the described amount of turkesterone, and it was generally expected that the amount of turkesterone would be higher than that of ecdysterone. However, our findings revealed that in two of the samples examined, the opposite was true. This finding is also consistent with a previous study by Kraiem et al. who reported that some supplements labeled as containing a higher dose of turkesterone actually contained higher doses of ecdysterone [17]. The differences between label and actual ecdysterone and turkesterone content highlight the lack of mandatory analytical control for dietary supplements in the European Union. The most important concern is that consumers are misled and cannot rely on good quality DSs without quality control and the introduction of (Good laboratory practice) GLP regulations, not only for medicinal products but also for DSs. It is worth noting that the sample that deviated from the labeled content was purchased online and not available for sale in pharmacies or drugstores.

The market for DSs has grown rapidly over the past two decades [47,48] and it is especially essential to ensure the quality control of these products. Supplementation with ecdysterone definitely provides some benefits, but these products must be quality controlled prior to marketing. Considering the positive effects of ecdysterone observed in rat studies, there has been increasing interest in its potential benefits for human consumption, Isenmann et al. conducted a study with human intake of 20-hydroxyecdysterone [13]. They observed a significantly greater increase in muscle mass in participants who received ecdysterone, with no significant increase in liver or kidney biomarkers of toxicity. They also reported that ecdysterone intake showed no significant changes in serum testosterone and luteinizing hormone levels. However, variations in insulin-like growth factor 1 concentrations were observed, along with temporal effects on estradiol and thyroxine levels after ecdysterone intake [13]. This emphasizes the effectiveness of ecdysterone supplementation in enhancing athletic performance. Studies by Parr et al. and Tsitsimpikou et. al. have shown that the metabolites of ecdysterone in human urine are deoxy-ecdysterone as the major metabolite and 2-deoxy-ecdysterone [27,49]. Parr et al. reported that when 50 mg of ecdysterone is ingested, the parent compound can be detected in urine even after two days, and the maximum concentration occurs 2–3 h after intake. Not only deactivated ecdysterone but also deoxy-ecdysterone, considered as its major metabolite, is present in urine [27]. Tsitsimpikou et. al. reported oral administration of 20 mg 20-hydroxyecdysterone and

detection of the parent and the metabolite after 21 h in urine [49]. Kraiem et al. tested 16 different ecdysterone supplements and found that only 5 contained quantifiable amounts of 20-hydroxyecdysterone. They selected 2 of the supplements (with higher and lower concentration of ecdysterone) for further studies and collected urine samples for up to 6 days. They found that at higher dose of ES, both the parent compound and its metabolite 14-deoxy-ecdysterone could be detected within 2 to 5 h post-administration, with 14-deoxy-ecdysterone still detectable up to 96 h and the parent compound up to 48 h. For the lower dose of ecdysterone, the metabolite 14-deoxy-ecdysterone could be detected up to 70 h and the parent drug up to 36 h [17]. Currently, turkesterone is not included in the monitoring program or doping lists of the WADA. However, due to the increasing use of supplements containing turkesterone and its anabolic properties, it is possible that it will be included in the future. Therefore, it would be appropriate to develop new, simple, and rapid methods for the analysis of turkesterone. Furthermore, to the best of our knowledge, the presented method is one of the shortest HPLC methods for the determination of turkesterone in DSs.

The development and validation of rapid and reliable methods for the determination of ecdysterone and turkesterone in DSs for professional athletes would be useful for future doping investigations [50]. High-performance liquid chromatography has proven to be a versatile, rapid, sensitive, and reproducible technique for both identification and quality control of doping substances [35,51–53]. In order to enhance future doping analysis, it is advisable to optimize the HPLC method for UHPLC-MS.

Although ecdysterone has demonstrated potential benefits in enhancing athletic performance and is currently monitored by the WADA, its potential inclusion on the Prohibited List for professional athletes could lead to certain challenges. Ecdysterone is naturally present in various foods (spinach, quinoa, and asparagus [54,55]) that are commonly present in the diet of many people. Consequently, WADA might consider prohibiting ecdysterone only in certain high doses, similar to other compounds that have dosage restrictions (e.g., inhaled salbutamol: maximum 1600 μg over 24 h in divided doses not exceeding 600 μg over 8 h starting at any dose; inhaled formoterol: maximum delivered dose of 54 μg over 24 h, etc.) [56]. Whether ecdysterone becomes a completely prohibited substance or is restricted to certain doses, or if WADA's experts determine that it is not a doping compound, it is crucial to monitor the DSs consumed by athletes for quality control. The present method in this study would provide accurate, sensitive, and rapid quantification of 20-hydroxyecdysterone and turkesterone in DSs.

4. Conclusions

The increasing availability of ecdysteroid-containing products on the market emphasizes the importance of developing simple and reliable analytical methods. In the current study, a reversed-phase HPLC method was proposed that could be used for the identification and quantification of ecdysterone and turkesterone simultaneously and separately, not only in dietary supplements, but also in plant extracts. The proposed method is simple, rapid, sensitive, and robust and is able to detect ecdysterone and turkesterone at low concentrations: 10.98 μg/mL and 11.43 μg/mL, respectively. It has been successfully applied to analyze dietary supplements containing ecdysterone and turkesterone. The developed method offers an accurate quantification of the substances present in dietary supplements, which is important for quality control and ensuring the reliability of the labeled contents. The present method could be used for both research and routine quality control.

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References

1. Dinan, L. Phytoecdysteroids: Biological Aspects. *Phytochemistry* **2001**, *57*, 325–339. [CrossRef] [PubMed]
2. Savchenko, R.G.; Veskina, N.A.; Odinokov, V.N.; Benkovskaya, G.V.; Parfenova, L.V. Ecdysteroids: Isolation, Chemical Transformations, and Biological Activity. *Phytochem. Rev.* **2022**, *21*, 1445–1486. [CrossRef]
3. Ecdybase (The Ecdysone Handbook)—A Free Online Ecdysteroids Database. Available online: https://ecdybase.org/ (accessed on 3 May 2023).
4. Bajguz, A.; Bąkała, I.; Talarek, M. Chapter 5—Ecdysteroids in Plants and Their Pharmacological Effects in Vertebrates and Humans. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, The Netherlands, 2015; Volume 45, pp. 121–145.
5. Arif, Y.; Singh, P.; Bajguz, A.; Hayat, S. Phytoecdysteroids: Distribution, Structural Diversity, Biosynthesis, Activity, and Crosstalk with Phytohormones. *Int. J. Mol. Sci.* **2022**, *23*, 8664. [CrossRef] [PubMed]
6. Kokoska, L.; Janovska, D. Chemistry and Pharmacology of Rhaponticum Carthamoides: A Review. *Phytochemistry* **2009**, *70*, 842–855. [CrossRef] [PubMed]
7. Mamarasulov, B.; Davranov, K.; Jabborova, D. Phytochemical, Pharmacological and Biological Properties of Ajuga Turkestanica (Rgl.) Brig (Lamiaceae). *Ann. Phytomedicine* **2020**, *9*, 44–57. [CrossRef]
8. Todorova, V.; Ivanov, K.; Delattre, C.; Nalbantova, V.; Karcheva-Bahchevanska, D.; Ivanova, S. Plant Adaptogens—History and Future Perspectives. *Nutrients* **2021**, *13*, 2861. [CrossRef] [PubMed]
9. Báthori, M.; Zupkó, I.; Hunyadi, A.; Gácsné-Baitz, E.; Dinya, Z.; Forgó, P. Monitoring the Antioxidant Activity of Extracts Originated from Various Serratula Species and Isolation of Flavonoids from Serratula Coronata. *Fitoterapia* **2004**, *75*, 162–167. [CrossRef] [PubMed]
10. Odinokov, V.N.; Galyautdinov, I.V.; Nedopekin, D.V.; Khalilov, L.M.; Shashkov, A.S.; Kachala, V.V.; Dinan, L.; Lafont, R. Phytoecdysteroids from the Juice of *Serratula Coronata* L. (Asteraceae). *Insect Biochem. Mol. Biol.* **2002**, *32*, 161–165. [CrossRef]
11. Guibout, L.; Mamadalieva, N.; Balducci, C.; Girault, J.-P.; Lafont, R. The Minor Ecdysteroids from *Ajuga Turkestanica*: Minor Ecdysteroids from *Ajuga Turkestanic* a. *Phytochem. Anal.* **2015**, *26*, 293–300. [CrossRef]
12. Tan, C.; Wang, J.; Li, X.; Meng, D.; Li, X. Constituents of phytosterone in Cyanotis arachnoidea C.B.Clark. *J. Shenyang Pharm. University* **2001**, *18*, 263–265.
13. Isenmann, E.; Ambrosio, G.; Joseph, J.F.; Mazzarino, M.; de la Torre, X.; Zimmer, P.; Kazlauskas, R.; Goebel, C.; Botrè, F.; Diel, P.; et al. Ecdysteroids as Non-Conventional Anabolic Agent: Performance Enhancement by Ecdysterone Supplementation in Humans. *Arch. Toxicol.* **2019**, *93*, 1807–1816. [CrossRef] [PubMed]
14. Tóth, N.; Szabó, A.; Kacsala, P.; Héger, J.; Zádor, E. 20-Hydroxyecdysone Increases Fiber Size in a Muscle-Specific Fashion in Rat. *Phytomedicine* **2008**, *15*, 691–698. [CrossRef] [PubMed]
15. Bathori, M.; Toth, N.; Hunyadi, A.; Marki, A.; Zador, E. Phytoecdysteroids and Anabolic-Androgenic Steroids—Structure and Effects on Humans. *Curr. Med. Chem.* **2008**, *15*, 75–91. [CrossRef] [PubMed]
16. Shuvalov, O.; Kirdeeva, Y.; Fefilova, E.; Netsvetay, S.; Zorin, M.; Vlasova, Y.; Fedorova, O.; Daks, A.; Parfenyev, S.; Barlev, N. 20-Hydroxyecdysone Confers Antioxidant and Antineoplastic Properties in Human Non-Small Cell Lung Cancer Cells. *Metabolites* **2023**, *13*, 656. [CrossRef]
17. Kraiem, S.; Al-Jaber, M.Y.; Al-Mohammed, H.; Al-Menhali, A.S.; Al-Thani, N.; Helaleh, M.; Samsam, W.; Touil, S.; Beotra, A.; Georgakopoulas, C.; et al. Analytical Strategy for the Detection of Ecdysterone and Its Metabolites in Vivo in UPA(+/+)-SCID Mice with Humanized Liver, Human Urine Samples, and Estimation of Prevalence of Its Use in Anti-doping Samples. *Drug Test Anal.* **2021**, *13*, 1341–1353. [CrossRef]
18. Pálinkás, Z.; Békési, D.; Utczás, M. Quantitation of Ecdysterone and Targeted Analysis of WADA-Prohibited Anabolic Androgen Steroids, Hormones, and Metabolic Modulators in Ecdysterone-Containing Dietary Supplements. *Separations* **2023**, *10*, 242. [CrossRef]
19. Haupt, O. *Recent Advances in Doping Analysis (20). Proceedings of the Manfred-Donike-Workshop, 30th Cologne Workshop on Dope Analysis, 26 February–2nd March 2012*; Sportverl Strauß: Köln, Germany, 2012.
20. Das, N.; Mishra, S.K.; Bishayee, A.; Ali, E.S.; Bishayee, A. The Phytochemical, Biological, and Medicinal Attributes of Phytoecdysteroids: An Updated Review. *Acta Pharm. Sin. B* **2021**, *11*, 1740–1766. [CrossRef]
21. Syrov, V.N.; Saatov, Z.; Sagdullaev, S.S.; Mamatkhanov, A.U. Study of the Structure—Anabolic Activity Relationship for Phytoecdysteroids Extracted from Some Plants of Central Asia. *Pharm. Chem. J.* **2001**, *35*, 667–671. [CrossRef]
22. Cahlíková, L.; Macáková, K.; Chlebek, J.; Hošťálková, A.; Kulhánková, A.; Opletal, L. Ecdysterone and Its Activity on Some Degenerative Diseases. *Nat. Prod. Commun.* **2011**, *6*, 1934578X1100600527. [CrossRef]
23. Ferro, N.; Tacoronte, J.E.; Reinard, T.; Bultinck, P.; Montero, L.A. Structure–Activity Analysis on Ecdysteroids: A Structural and Quantum Chemical Approach Based on Two Biological Systems. *J. Mol. Struct. Theochem.* **2006**, *758*, 263–274. [CrossRef]

24. Dinan, L. Ecdysteroid Structure-Activity Relationships. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Bioactive Natural Products (Part J); Elsevier: Amsterdam, The Netherlands, 2003; Volume 29, pp. 3–71.
25. The WADA 2020 Monitoring Program. Available online: https://www.Wada-Ama.Org/Sites/Default/Files/Wada_2020_english_monitoring_program_.Pdf (accessed on 3 May 2023).
26. The WADA 2023 Monitoring Program. Available online: https://www.Wada-Ama.Org/Sites/Default/Files/2022-09/2023list_monitoring_program_en_final_9_september_2022.Pdf (accessed on 3 May 2023).
27. Parr, M.K.; Ambrosio, G.; Wuest, B.; Mazzarino, M.; De La Torre, X.; Sibilia, F.; Joseph, J.F.; Diel, P.; Botrè, F. *Targeting the Administration of Ecdysterone in Doping Control Samples*; Pharmacology and Toxicology: Tucson, AZ, USA, 2019.
28. Grucza, K.; Wicka, M.; Drapała, A.; Kwiatkowska, D. Determination of Ecdysterone in Dietary Supplements and Spinach by Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Separations* **2022**, *9*, 8. [CrossRef]
29. Ecdysteroid-Containing Food Supplements from Cyanotis Arachnoidea on the European Market: Evidence for Spinach Product Counterfeiting | Scientific Reports. Available online: https://www.nature.com/articles/srep37322 (accessed on 4 May 2023).
30. Timofeev, N.P.; Koksharov, A.V. Study of Leuzea Leuzea from Leaves: Results of 15 Years of Trials in Athletics. *New Unconv. Plants Prospect. Use* **2016**, *12*, 502–505.
31. Wu, J.; Gao, L.; Shang, L.; Wang, G.; Wei, N.; Chu, T.; Chen, S.; Zhang, Y.; Huang, J.; Wang, J.; et al. Ecdysterones from Rhaponticum Carthamoides (Willd.) Iljin Reduce Hippocampal Excitotoxic Cell Loss and Upregulate MTOR Signaling in Rats. *Fitoterapia* **2017**, *119*, 158–167. [CrossRef] [PubMed]
32. Xia, X.; Zhang, Q.; Liu, R.; Wang, Z.; Tang, N.; Liu, F.; Huang, G.; Jiang, X.; Gui, G.; Wang, L.; et al. Effects of 20-Hydroxyecdysone on Improving Memory Deficits in Streptozotocin-Induced Type 1 Diabetes Mellitus in Rat. *Eur. J. Pharmacol.* **2014**, *740*, 45–52. [CrossRef] [PubMed]
33. Krasutsky, A.G.; Cheremisinov, V.N. The Use of Levzey's Extract to Increase the Efficiency of the Training Process in Fitness Clubs Students. In Proceedings of the Actual Problems of Biochemistry and Bioenergy of Sport of the XXI Century, Moscow, Russia, 10–26 April 2017; pp. 382–388.
34. Garthe, I.; Maughan, R.J. Athletes and Supplements: Prevalence and Perspectives. *Int. J. Sport Nutr. Exerc. Metab.* **2018**, *28*, 126–138. [CrossRef] [PubMed]
35. Kozhuharov, V.R.; Ivanov, K.; Ivanova, S. Dietary Supplements as Source of Unintentional Doping. *BioMed. Res. Int.* **2022**, *2022*, e8387271. [CrossRef]
36. Vovk, I.; Glavnik, V. Chapter 21—Analysis of Dietary Supplements. In *Instrumental Thin-Layer Chromatography*; Poole, C.F., Ed.; Elsevier: Boston, MA, USA, 2015; pp. 589–635, ISBN 978-0-12-417223-4.
37. Gafner, S.; Blumenthal, M.; Foster, S.; Cardellina, J.H.I.; Khan, I.A.; Upton, R. Botanical Ingredient Forensics: Detection of Attempts to Deceive Commonly Used Analytical Methods for Authenticating Herbal Dietary and Food Ingredients and Supplements. *J. Nat. Prod.* **2023**, *86*, 460–472. [CrossRef]
38. Sharma, M.K.; Koppisetti, H.P.; Lohar, P.; Maheshwari, R.; Sengupta, P.; Tekade, R.K. Chapter 23—Understanding the Connection between Dietary Supplementation and Inadvertent Doping. In *Essentials of Pharmatoxicology in Drug Research*; Tekade, R., Ed.; Advances in Pharmaceutical Product Development and Research; Academic Press: Cambridge, MA, USA, 2023; Volume 1, pp. 599–623, ISBN 978-0-443-15840-7.
39. Sadia, M.; Mahmood, A.; Ibrahim, M.; Irshad, M.K.; Quddusi, A.H.A.; Bokhari, A.; Mubashir, M.; Chuah, L.F.; Show, P.L. Microplastics Pollution from Wastewater Treatment Plants: A Critical Review on Challenges, Detection, Sustainable Removal Techniques and Circular Economy. *Environ. Technol. Innov.* **2022**, *28*, 102946. [CrossRef]
40. EMA ICH Q2(R2) Validation of Analytical Procedures—Scientific Guideline. Available online: https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline (accessed on 7 December 2022).
41. Poojari, R. Phytochemical Fingerprinting, Cytotoxic, Antimicrobial, Antitubercular, Antimycotic Potentials of Sida Rhombifolia Subsp. Retusa and Embelia Tsjeriam-Cottam. *Asia Pac. Life Sci. Compend.* **2011**, *4*, 201–214.
42. Liktor-Busa, E.; Báthori, M. Analysis of the Ecdysteroid Profile of Serratula Wolffii Roots. Ph.D. Thesis, University of Szeged, Szeged, Hungary, 2008.
43. Głazowska, J.; Kamiński, M.M.; Kamiński, M. Chromatographic Separation, Determination and Identification of Ecdysteroids: Focus on Maral Root (*Rhaponticum Carthamoides, Leuzea Carthamoides*). *J. Sep. Sci.* **2018**, *41*, 4304–4314. [CrossRef]
44. Dinan, L.; Harmatha, J.; Lafont, R. Chromatographic Procedures for the Isolation of Plant Steroids. *J. Chromatogr. A* **2001**, *935*, 105–123. [CrossRef]
45. Ambrosio, G.; Wirth, D.; Joseph, J.F.; Mazzarino, M.; de la Torre, X.; Botrè, F.; Parr, M.K. How Reliable Is Dietary Supplement Labelling?-Experiences from the Analysis of Ecdysterone Supplements. *J. Pharm. Biomed. Anal.* **2020**, *177*, 112877. [CrossRef]
46. Lafont, R.; Dinan, L. Practical Uses for Ecdysteroids in Mammals Including Humans: And Update. *J. Insect Sci.* **2003**, *3*, 7. [CrossRef]
47. Petkova-Gueorguieva, E.S.; Getov, I.N.; Ivanov, K.V.; Ivanova, S.D.; Gueorguiev, S.R.; Getova, V.I.; Mihaylova, A.A.; Madzharov, V.G.; Staynova, R.A. Regulatory Requirements for Food Supplements in the European Union and Bulgaria. *Folia. Med. (Plovdiv.)* **2019**, *61*, 41–48. [CrossRef]
48. Lam, M.; Khoshkhat, P.; Chamani, M.; Shahsavari, S.; Dorkoosh, F.A.; Rajabi, A.; Maniruzzaman, M.; Nokhodchi, A. In-Depth Multidisciplinary Review of the Usage, Manufacturing, Regulations & Market of Dietary Supplements. *J. Drug Deliv. Sci. Technol.* **2022**, *67*, 102985. [CrossRef]

49. Tsitsimpikou, C.; Tsamis, G.D.; Siskos, P.A.; Spyridaki, M.-H.E.; Georgakopoulos, C.G. Study of Excretion of Ecdysterone in Human Urine. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1796–1801. [CrossRef]
50. De Cock, K.J.S.; Delbeke, F.T.; Van Eenoo, P.; Desmet, N.; Roels, K.; De Backer, P. Detection and Determination of Anabolic Steroids in Nutritional Supplements. *J. Pharm. Biomed. Anal.* **2001**, *25*, 843–852. [CrossRef]
51. Parys, W.; Dołowy, M.; Pyka-Pająk, A. Significance of Chromatographic Techniques in Pharmaceutical Analysis. *Processes* **2022**, *10*, 172. [CrossRef]
52. Parys, W.; Dołowy, M.; Pyka-Pająk, A. Current Strategies for Studying the Natural and Synthetic Bioactive Compounds in Food by Chromatographic Separation Techniques. *Processes* **2021**, *9*, 1100. [CrossRef]
53. Munir, M.; Saeed, M.; Ahmad, M.; Waseem, A.; Alsaady, M.; Asif, S.; Ahmed, A.; Shariq Khan, M.; Bokhari, A.; Mubashir, M.; et al. Cleaner Production of Biodiesel from Novel Non-Edible Seed Oil (*Carthamus Lanatus* L.) via Highly Reactive and Recyclable Green Nano CoWO3@rGO Composite in Context of Green Energy Adaptation. *Fuel* **2023**, *332*, 126265. [CrossRef]
54. Thiem, B.; Kikowska, M.; Maliński, M.P.; Kruszka, D.; Napierała, M.; Florek, E. Ecdysteroids: Production in Plant in Vitro Cultures. *Phytochem. Rev.* **2017**, *16*, 603–622. [CrossRef] [PubMed]
55. Dinan, L.; Balducci, C.; Guibout, L.; Lafont, R. Database of Human Food Plants and Whether They Have Been Assessed for the Presence or Absence of Ecdysteroids. Available online: https://ecdybase.org/ecdysteroids_in_food_plants.pdf (accessed on 22 May 2023).
56. The Wada 2023 Prohibitet List. Available online: https://www.Wada-Ama.Org/Sites/Default/Files/2023-05/2023list_en_final_9_september_2022.Pdf (accessed on 22 May 2023).