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Determination of Withanolides and Withanosides in Ashwagandha Based Products Using HPLC-Drift-Tube-Ion-Mobility Quadrupole Time-of-Flight Mass Spectrometry

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ABSTRACT

A total of 19 products based on extracts from *Withania somnifera* (L.) Dunal, better known by its more common name ashwagandha, and five products based on ashwagandha root powder were analyzed with respect to their content in the biologically relevant substances belonging to the group of withanolides and withanosides. Using HPLC coupled to drift-tube ion-mobility quadrupole time-of-flight mass spectrometry (DT-IM-QTOF-MS), 19 withanolides and withanosides could be tentatively identified. The comparison of the results from the quantitative analysis with the information on the product labels showed that the percentage of withanolides and withanosides deviated from the stated specifications by at least a factor of two and at most a factor of 35.

1 | Introduction

Medicines derived from plants have played an important role in preserving health and handling disease throughout history [1, 2]. In several Asian and African countries, *Withania somnifera* (L.) Dunal, widely employed in these regions within traditional medicine, can be regarded as a prominent example supporting this assumption. Particularly in Ayurveda, *Withania somnifera*, one of the oldest traditional medicines in India and better known by its popular name "ashwagandha", has a long history of application and is frequently used for a variety of indications [3, 4]. Although widely employed in large parts of the world, for a long time ashwagandha has been rather unknown and exotic in Europe, with no history of usage in western (traditional) medicines. In the past years, this has changed drastically and nowadays a multitude of ashwagandha-based products (primarily marketed as nutritional supplement) were launched in Europe and the United States in order to satisfy the increasing interest and demand. Thereby, the more traditional formulations or modes of preparation (which are often time consuming) were adapted to western lifestyle by manufacturing tablets or capsules, allowing an easier intake of this product. Ashwagandha is primarily used due to its diverse pharmacological properties, including anti-inflammatory, antioxidant, immunomodulatory, and adaptogenic effects, whereby these therapeutic benefits are particularly attributed to the presence of bioactive compounds called withanolides (a group of steroidal compounds) and withanosides (gylcosides of withanolides and therefore saponins)

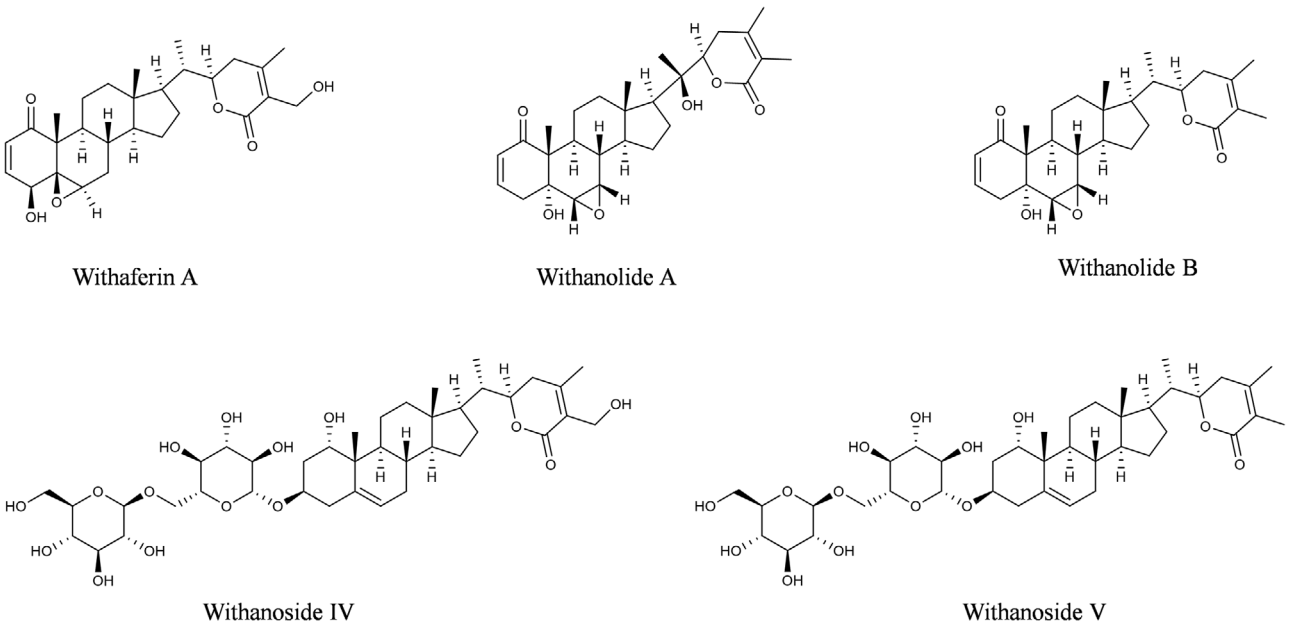

FIGURE 1 | Structures of five relvant withanolides and withanosides.

[5–9]. Although a large number of withanolides and withanosides are known [10], and more than 40 compounds belonging to this class have been identified in concentrated extracts of *W. somnifera* [11], ashwagandha (root) extracts are mostly characterized by the dominating species, comprising around 10 representatives of this group of substances [12]. This is, for example, reflected in some of the official methods for analyzing *W. somnifera* plant material based on thin layer chromatography (TLC) and HPLC with UV detection [13, 14].

Focusing on reports dealing with the analysis of withanolides and withanosides for characterizing ashwagandha products, a substantial number of research papers proposing analytical procedures for the detection and quantitation of these molecules in the different parts of *W. somnifera* plants as well as in processed products (primarily dried and powdered) can be found in the literature (for an exemplary review, see [15]). These comprise analytical approaches employing nuclear magnetic resonance (NMR) [9, 16], TLC [17–19], HPLC with UV [20–22], and/or evaporative light scattering detection [23], and finally, also HPLC coupled to more information rich detectors such as for mass spectrometry (MS) [10, 24]. Thereby, primarily plants parts from *W. somnifera* are qualitatively and sometimes also quantitatively investigated with respect to the presence of withanolides and withanosides.

In this work, we present the analysis of more than 20 commercially available ashwagandha-based products sold in the form of capsules, filled with standardized and non-standardized ashwagandha extracts in combination with a variety of additives and sometimes also ashwagandha root powder. Using HPLC coupled to drift-tube ion-mobility quadrupole time-of-flight mass spectrometry (DT-IM-QTOF-MS), we could tentatively identify 19 withanolides and withanosides in these formulations by measuring accurate mass, determination of collision cross sections (CCS), MS^2 experiments, and (if available) comparison with standard substances. Quantitative analysis was performed in order to evaluate especially those products advertised as highly potent ashwagandha extracts, claiming to contain exceptionally high amounts of the biologically relevant ingredients. Thereby, the overall content in withanolides and withanosides (often manufacturers do not really differentiate between the two groups and simply use "total withanolide content") specified on the product label was compared to the data obtained from our analyses.

2 | Experimental

2.1 | Chemicals and Materials

The standard mix used for analyte identification and quantification was "ashwagandha dietary ingredients mix solution" purchased from Merck (Darmstadt, Germany). The 1 mL ampule contains a defined amount of five withanolides and withanosides in methanol: 100 mg/L withaferin A, 100 mg/L withanolide A, 20 mg/L withanolide B, 100 mg/L withanoside IV, and 100 mg/L withanoside V (for structures, please see Figure 1). The purchased standard mix has been diluted with methanol to obtain standards in the concentration range from 0.01 to 10 mg/L for withaferin A, withanolide A, withanoside IV, and withanoside V and from 0.002 to 2 mg/L for withanolide B. Withanolides and withanosides not present in the standard mix were quantified using the calibration of compounds that were present in the "ashwagandha dietary ingredients mix solution."

Acetonitrile and methanol (HPLC grade) were purchased from VWR (Vienna, Austria). Formic acid and ammonium formate (both for LC-MS) were purchased from Merck (Darmstadt, Germany).

Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Ashwagandha root was procured from a pharmaceutical wholesaler (Kottas Pharma GmbH) who supplies Austrian pharmacies

with medicinal plants. The specimen with the batch number W23201745, taken in accordance with GMP regulations, was checked and confirmed for identity by botanically trained experts. The root pieces were finely ground before extraction. Four different root powder samples (two bulk materials and two capsule products) and 19 different samples of commercially available dietary supplements labeled as ashwagandha root extracts were obtained from drug stores, pharmacies and online stores. To avoid potential conflicts, information about the manufacturers of products that do not meet their stated claims is deliberately omitted. Table S1 gives an overview of the investigated samples (including withanolide and withanoside content based on information from the manufacturers ingredient list).

2.2 | Preparation of Extracts

Note that 100 mg of each ashwagandha root powder and dietary supplement was suspended in 15 mL of methanol and subjected to extraction at 70°C for 30 min in a closed vial using an ultrasonic bath. After centrifugation, the supernatant was collected, and the procedure was repeated twice with the remaining solid. The three supernatant fractions from these extractions were combined, and the total volume was adjusted to 50 mL in a volumetric flask. In summary, the samples (roots and dietary products labeled as root extracts) were extracted three times with methanol at elevated temperatures. In a fourth extraction, only negligible amounts of the investigated compounds were detected, indicating that triple extraction is clearly sufficient.

2.3 | HPLC-DT-IM-QTOF-MS

For compound separation, a reversed-phase (RP) HPLC (Agilent 1260, Agilent Technologies, Waldbronn, Germany) was equipped with a YMC Triart C18 column (3 × 100 mm, 3 µm particle size). The mobile phase comprised acetonitrile (solvent A), water (solvent B), 0.1% formic acid, and 10 mM ammonium formate in water (solvent C). Initial conditions of the gradient program were 25% solvent A, 70% solvent B, and 5% solvent C. Solvent C was kept constant at 5% throughout the whole chromatographic run. The ratio of solvents changed to 55% solvent A, 40% solvent B, and 5% solvent C at 10.0 min, then to 95% solvent A, 0% solvent B, and 5% solvent C at 13.0 min, which was maintained until 16 min. The conditions returned to their initial conditions at 16.1 min, completing the run within a total time of 20 min. The flow rate was maintained at 0.5 mL/min. The injection volume was set to 5 µL. Samples with very high concentrations of withaferin A were also injected using 1 µL to fit the calibration range. The chosen UV detection wavelength was 227 nm. Standards and samples were always analyzed in triplicate.

The HPLC system was coupled to a high-resolution DT-IM-quadrupole time-of flight (DT-IM-QTOF)-MS (Agilent 6560, Agilent Technologies, Waldbronn, Germany) equipped with an Agilent Dual Jet Stream Electrospray Ionization source, which was operated in positive ion mode. The drying/sheath gas temperature was 350°C, the drying gas flow rate was 9.5 L/min, the sheath gas flow rate was 11 L/min (both nitrogen), the nebulizer pressure was 35 psi, the capillary voltage was 3750 V, the nozzle voltage was 1000 V, and the fragmentor voltage was 400 V.

The DT-IM-QTOF-MS was tuned in the "fragile ion" mode and operated with 5-bit multiplexing. The settings were as follows: trap fill time was 1800 µs, trap release time was 250 µs, frame rate was 0.9 frames s^{-1}, transient rate was 17 transients $frame^{-1}$, and maximum drift time was 60 ms. Under advanced parameters following settings were set: drift tube entrance voltage was 1567 V, drift tube exit voltage was 217 V, rear funnel entrance was 210.5 V, and rear funnel exit was 38 V.

2.4 | Data Processing

Data evaluation/processing was done by using Agilent MassHunter Qualitative Analysis B.07.00, PNNL PreProcessor 4.0 (2022.02.18), IM-MS Browser 10.0.1, and Agilent MassHunter Workstation Mass Profiler 10.0.2.

The PNNL PreProcessor software was used for demultiplexing the ion-mobility (IM) data files. The data were calibrated with the recorded single field tune using IM-MS browser. The drift times and the $^{DT}CCS_{N2}$ were determined by performing a feature extraction.

Principal component analysis (PCA) was performed with the aim to reduce the number of 19 found withanolides and withanosides and their quantities characterizing the products to just two principal components. This has been carried out using the online statistical analysis tool of MetaboAnalyst 6.0 (https://www.metaboanalyst.ca, accessed on August 26, 2024) [25]. Missing values were estimated by the online platform. Data normalization, transformation, and scaling was omitted to relate the absolute quantities of the particular analytes in the products to each other.

3 | Results and Discussion

3.1 | Analysis of Ashwagandha Products

Twenty-one commercially available ashwagandha preparations (all marketed in the form of capsules) were investigated with respect to their content in withanolides and withanosides. For comparison, ashwagandha root powder, either purchased from a manufacturer or lab made by grinding ashwagandha root parts, was also analyzed. Thereby, a series of analytical methods were employed. The capsule content was inspected optically using transmitted light microscopy (for details on microscopy, see [26]), subsequently the material was extracted or dissolved in an appropriate solvent and subjected to RP-HPLC with UV and DT-IM-QTOF-MS detection.

3.2 | Detection of Withanolides and Withanosides Using HPLC DT-IM-QTOF-MS

Using HPLC-DT-IM-QTOF-MS, 19 withanolide-related substances and their glucosides could be (tentatively) identified. Thereby, for withaferin A, withanolide A, withanolide B, withanoside IV, and withanoside V, actual identification was possible as standards for these substances were available for comparison. For the other compounds, 27-hydroxywithanone,

TABLE 1 | Retention times, accurate mass, and collision cross sections determined for the 19 withanolide- and withanoside-related compounds.

Analyte number	Compound	RT (min])	m/z $[M + H]^+$	$^{DT}CCS_{N2}$ (Å^2)	$^{DT}CCS_{N2}$ rsd (%)
1	Withanoside IV	5.57	783.4161	281.9	0.38
2	27-Hydroxywithanone	5.65	487.2690	n.d.	n.d.
3	Withanoside VII	6.22	783.4161	275.8	0.26
4	Viscosalactone B isomer 1	6.59	489.2847	228.8	0.32
5	Viscosalactone B isomer 2	6.71	489.2847	228.3	0.27
6	Viscosalactone B isomer 3	6.92	489.2847	228.5	0.31
7	Viscosalactone B isomer 4	7.78	489.2847	225.0	0.37
8	Dihydrowithaferin A isomer 1	8.07	473.2898	216.5	0.22
9	Dihydrowithaferin A isomer 2	8.40	473.2898	225.7	0.25
10	Withanoside V	8.69	767.4212	281.1	0.23
11	Withaferin A	9.09	471.2741	224.8	0.20
12	12-deoxywithastramonolide	9.77	471.2741	217.3	0.25
13	Withanone	10.04	471.2741	217.2	0.24
14	Withanolide A	10.55	471.2741	215.0	0.32
15	Withanolide B isomer 1	11.23	455.2795	222.1	0.35
16	Withanolide B isomer 2	11.87	455.2795	n.d.	n.d.
17	Withanolide B isomer 3	12.33	455.2795	223.5	0.12
18	Withanolide B isomer 4	13.75	455.2795	219.8	0.25
19	Withanolide B	13.87	455.2795	217.8	0.26

n.d. not detected due to insufficient signal intensities.

viscosalactone B, withanoside VII, dihydrowithaferin A, 12-deoxywithastramonolide, and withanone as well as for the isomers detected for these and the before mentioned substances, tentative identification was based on retention time, accurate mass, and determination of CCS. For some of the molecules (due to low concentrations), the measurement of meaningful CCS values was not possible. For all others, this parameter could be determined with excellent reproducibility (RSD values below 0.4%). An overview of the results from these measurements is given in Table 1. In most cases (as expected), CCS values do not significantly differ for the isomers. In one case, namely, for the two dihydrowithaferin A isomers detected (showing slightly different retention times), CCS values differing by more than 4% were observed for the protonated species. Despite a similar trend was observed when comparing the respective values for the Na^+ adducts, it was less pronounced. MS × MS experiments did not show significant differences between the two isomers. The set of withanolides and withanosides found in ashwagandha root extracts is in accordance with the literature published so far.

3.3 | Quantitative Analysis Using HPLC-UV and HPLC DT-IM-QTOF-MS

Starting point for the investigation on the quantitative determination of withanolides and withanosides not only in ashwagandha root and root powder but also in commercially available products (in particular in the form of capsules) was a method reported in the United States Pharmacopeia (USP) [12], the AOAC method [11], as well as a procedure taken from the literature [10]. Within these papers different strategies for the quantitation of withanolides and withanosides are pursued. Approaches for quantitation employed range from the USP method, where withaferin A is used as the standard for all withanolides and withanoside IV for all withanolides, over the method described by AOAC with six standard substances to a paper by Girme et al. [12] employing a set of 11 standards for quantitation of the investigated compounds. For the latter, as several compounds were not available commercially, standards extracted and purified in-lab had to be employed. In our study, we pursued a middle way, whereby quantitative evaluation is based on the use of withaferin A, withanolide A, withanolide B, withanoside IV, and withanoside V as standard substances. Subsequently, withanoside IV was employed for the quantitation of withanoside VII. Withanolide B calibration was used for all withanolide B isomers, and withaferin A calibration was used for all other compounds.

First, a series of experiments was performed to evaluate whether quantitation based on signals from UV detection and those from detection by DT-IM-QTOF-MS led to significantly different results. As this was not the case, further work was limited to the use of DT-IM-QTOF-MS. In Table 2, an overview of the results from quantitative analysis of the 24 samples (five root powders and 19 products based on ashwagandha extracts) is provided. The products based on ashwagandha extracts should, according to the specification given on the label and in the more detailed description provided in the smallprint, show a totaled content of withanolides and withanosides (% age already corrected for non-ashwaghanda-related ingredients) in the range of 1.4% (product 14) to 9.8% (product 22). As can be seen from Table 2 and

TABLE 2 | Overview of the results from quantitative analysis of the 24 samples.

	Withanolides (mg g^{-1})	Withanosides (mg g^{-1})	wt.% ∑ Withanolides and withanosides
Product 1	1.32	1.23	0.26
Product 2	1.25	1.04	0.23
Product 3	1.02	1.02	0.20
Product 4	0.82	0.93	0.17
Product 5	1.29	0.71	0.20
Product 6	1.35	0.30	0.16
Product 7	0.71	0.13	0.08
Product 8	1.18	0.27	0.15
Product 9	1.24	0.27	0.15
Product 10	0.95	0.24	0.12
Product 11	0.69	0.45	0.11
Product 12	0.88	0.28	0.12
Product 13	0.74	0.22	0.10
Product 14	0.94	0.63	0.16
Product 15	8.10	5.07	1.32
Product 16	5.70	4.62	1.03
Product 17	0.66	0.76	0.14
Product 18	0.64	0.25	0.09
Product 19	0.96	0.22	0.12
Product 20	1.71	0.98	0.27
Product 21	25.12	8.03	3.31
Product 22	3.41	1.03	0.44
Product 23	1.02	1.09	0.21
Product 24	13.42	7.01	2.04

in more detail, listing values for all detected withanolides and withanosides, from Table S2 almost none of the investigated samples came even close to the values provided by the product label. Only the products containing exclusively root powder could fulfill the promises made.

This can also be seen from Figure 2 showing a PCA of the found withanolide and withanoside compounds and their content in the analyzed samples, where the majority of products cluster with the root powder samples (found in the left lower corner) and only four samples (15, 16, 21, and 24) show a distinctly different position. Thereby, products 15, 16 and 21 stand out due to their much higher content in withanolides and withanosides and are located at the largest distance from the "rootpowder cluster." This is substantially caused by a very high concentration of withaferin A with this compound standing for 70% of the total content in withanolides in the case of 16, 66% for product 15, and 64% for product 21. This can also be seen in Figure 3, providing a comparison of the extracted ion chromatograms obtained for a root powder sample and three extract-based products—showing

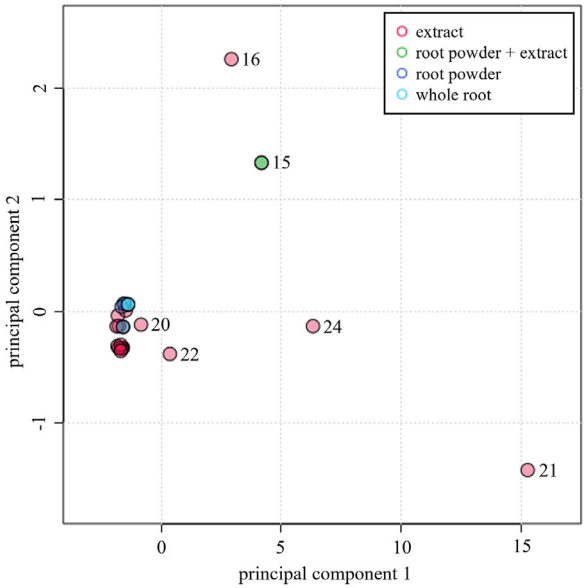

FIGURE 2 | PCA plot comparing the quantities of withanolides and withanosides in the analyzed products. Labels refer to the product numbers; color-coding according to the sample content. This figure was created using MetaboAnalyst 6.0 [23].

the prominent peak for withaferin A for both products (16 and 21). Particularly for product 16 (trace C), this might be explained by the use of not only the roots but also aerial parts of the plant that commonly contain much higher concentrations of withaferin A. Product 21 (trace D) shows increased signal intensities for most of the other compounds leading to a total content of 3.3% withanolides and withanosides, which is the highest of all products investigated (although still only a bit more than half of the 6% specified on the label). An interesting fact in this context is that several of the manufacturers advertise their products as containing only low amounts or even no withaferin A at all (for most products the percentage of withaferin A within the group of withanolides lies between 12 and 35), although there are no studies relating this substance to actual health issues [27, 28]. When comparing traces A and B, the high degree of similarity between the two chromatograms is striking. This is in contrast to what would be expected when comparing the information provided by the manufacturers. Trace A shows the analysis of milled root powder (product 1)—containing 0.2%–0.3% of withanolides and withanosides (a value complying well with data from the literature as well as from the US pharmacopeia). On the other hand, trace B depicts a chromatogram obtained from the analysis of an extract based product (product 6) that should (according to the label) contain a 15–20 times higher amount of withanolides and withanosides.

4 | Conclusions

In the present work, we analyzed 19 products (all sold in the form capsules) including Ashwagandha extracts with respect to their content in the biologically relevant ingredients (withanolides and withanosides) and subsequently compared the results to simple Ashwagandha root powder. Despite only a fraction of the plethora of ashwagandha-based dietary supplements marketed via various

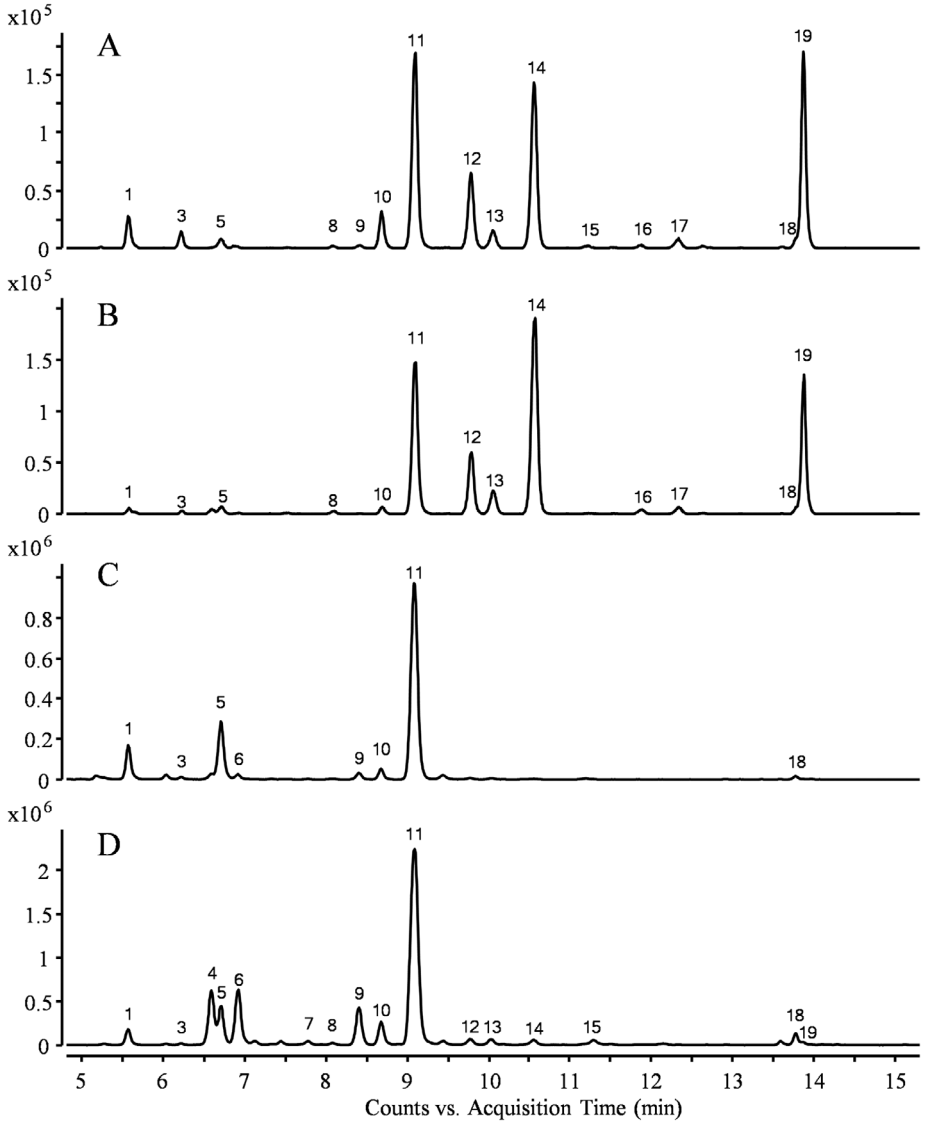

FIGURE 3 | Extracted ion chromatograms for four investigated ashwagandha related samples. Trace A—ashwagandha root powder (product 1); trace B—ashwagandha extract based product (product 6); trace C—ashwagandha extract based product (product 16); trace D—ashwagandha extract based product (product 21). Peak labeling: see Table 1.

sources (from pharmacies over drug stores to the internet) has been the subject of this study, the outcome may give some insight into the situation within the market. Our analyses revealed that none of the tested samples complied with the data provided by the manufacturer when it comes to the percentage of withanolides and withanosides present in these formulations. Although some of the products failed by a factor <4 only, the majority showed concentrations of these biologically relevant ingredients which were on average lower by a factor >30. As a consequence, a more thorough quality control of these dietary supplements, particularly when it comes to comparing the actual content in relevant ingredients with that stated by the manufacturer, might be an option for the future.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.