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ORIGINAL ARTICLE

Determination of adulterants in whey protein food supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry

Determinação de adulterantes em proteína de soro de leite por cromatografia líquida acoplada à espectrometria de massas de alta resolução do tipo Orbitrap

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Abstract

Liquid chromatography coupled to Orbitrap high resolution mass spectrometry was shown to be an adequate technique to control the adulteration of whey protein food supplements with prohibited substances, not declared on the labels. An extraction method combined with an instrumental analysis that allowed for the determination of 105 substances in whey protein food supplements, was established. The pre-treatment of the samples consisted of protein precipitation and solid-phase extraction using weak cation exchange functionalized polymeric sorbent cartridges. The samples were directly analyzed by LC-Orbitrap-HRMS. The selectivity, limit of detection, repeatability, recovery, carryover and matrix effect were estimated as the validation parameters. The repeatability obtained was 96.19% and the recovery 83.80%, but carryover and the matrix effect were not observed. The present method was successfully applied to the analysis of commercial samples, verifying adulteration by diuretics (conivaptan and politiazide) and a stimulant (benfluorex) in seven of the eleven brands evaluated.

Keywords: Whey protein food supplement; Adulterants; Pharmacological action; Stimulants; Diuretics; Orbitrap; Method validation.

Resumo

A cromatografia líquida acoplada à espectrometria de massas de alta resolução do tipo Orbitrap demonstrou ser uma técnica adequada para o controle de adulteração de substâncias proibidas não declaradas nos rótulos de suplementos proteicos derivados de soro de leite. Foi estabelecido um método de extração combinado a uma análise instrumental que permitiu a determinação de 105 substâncias em suplemento proteico derivado de soro de leite. O pré-tratamento das amostras consistiu na precipitação de proteínas e na extração em fase sólida, utilizando-se cartuchos com sorventes poliméricos baseados em troca catiônica. As amostras foram diretamente analisadas por CL-EMAR-Orbitrap. Foram estimados, como parâmetros de validação, seletividade, limite de detecção, repetitividade, recuperação, arraste e efeito de matriz. A repetitividade obtida foi de 96,19% e a recuperação foi de 83,80%. Arraste e efeito de matriz não foram observados. O presente método foi aplicado com sucesso na análise de amostras comerciais, nas quais foram verificadas adulterações, em sete das 11 marcas avaliadas, em diuréticos (conivaptan e politiazida) e estimulante (benfluorex).

Palavras-chave: Suplemento alimentar de proteína de soro de leite; Adulterantes; Ação farmacológica; Estimulantes; Diuréticos; Orbitrap; Validação de método.

1 Introduction

Over the years, technological and scientific advancement for improving human performance have been studied in different areas (Andrade et al., 2019; Roco & Bainbridge, 2002; Thomas et al., 2015). However, nutrition is still considered the most relevant aspect regarding muscle building, endurance and strength (Bagchi et al., 2013; McClung & Murray-Kolb, 2013; Pritchard-Peschek et al., 2013). Due to progress in this area, many athletes, non-athletes and patients with different diseases have been using functional foods to improve their health (Fayh et al., 2013; Horikawa et al., 2013; Mathews, 2018; Maughan et al., 2018; Rondanelli et al., 2016).

The class of food supplements most widely used in the world is that of milk constituents named Whey Protein Food Supplement (WPFS) (Chen et al., 2014; Fayh et al., 2013). The WPFS is obtained from the preparation of cheese, specifically during the casein precipitation step (milk protein) in which it forms a supernatant, the milk serum (Aquino et al., 2017; Chen et al., 2014; Garrido et al., 2016). WPFS shows the following properties: increase in resistance, muscle hypertrophy and decreased body fat (Andrade et al., 2019; Chen et al., 2014; Frestedt et al., 2008; Garrido et al., 2016).

There is no compatible regulation for WPFS between countries (Neves & Caldas, 2015), and the absence of a specific regulation and better monitoring in the manufacturing process of food supplements, may result in incompatibilities related to the label and content (Andrade et al., 2019; Parra et al., 2011). These mismatches could be related to the quantities of nutrients or other components described on the label or to the presence of substances (intentionally added) that are not reported, resulting in adulteration issues (Andrade et al., 2019; Marcus, 2016).

There are many studies on the adulteration of supplements, and more attention has been focused on adulteration by substances that have pharmacological properties (Garrido et al., 2016; Lu et al., 2010; Martínez-Sanz et al., 2017; Müller et al., 2018; Woo et al., 2013). The main cases of food supplement adulteration are related to the following classes: anabolic agents (provide increases in muscle mass and decreases in body fat); diuretics (decrease body liquids and mask the presence of other substances in the sample); and stimulants (weight loss, increase alertness and reduce fatigue) (Hernandez & Nahas, 2009; Müller et al., 2018; Neves & Caldas, 2015; Martínez-Sanz et al., 2017). Martello et al. (2007), described a qualitative liquid chromatography tandem mass spectrometry (LC-MS/MS) method used to detect the following anabolic androgenic steroids (4-androsten-3,17-dion, 4-oestren-3,17-dion, 5α-androsten-17β-ol-3-one, boldenone, nandrolone, nandrolone decanoate, testosterone and testosterone decanoate) and ephedrine

in food supplements. The LC-MS/MS analysis was carried out using selected reaction monitoring (SRM) in an ion-trap system equipped with an atmospheric pressure chemical ionization (APCI) probe operating in the positive-ion mode. However, this is a target method for a limited number of substances. The method was applied to 64 nutritional supplements and a total of 12.5% of the nutritional supplements analyzed contained banned substances not declared on the label (anabolic steroids and ephedrine) (Martello et al., 2007). However, some relevant classes of substances such as diuretics and anorectic agents were not evaluated, probably because analysis by SRM only in the positive ionization mode does not allow for the scanning of a comprehensive number of substances. Moreover, for the LC-MS/MS analysis, the liquid-liquid extraction sample preparation using n-pentane and diethyl ether, limited the extraction of acid analytes. In 2010, Lu et al., described a sensitive and specific liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS) method for the analysis of 18 drugs used in the treatment of hypertension, including diuretics, as adulterants in dietary supplements (Lu et al., 2010). However, once again it was a very limited procedure regarding the number of substances analyzed.

Multi-target procedures need a more elaborate analytical method, and usually include an ESI interface operating in both positive and negative ionization modes. Moreover, extraction and matrix effects are also a vulnerable point for the routine inspection of WPFS by a single and comprehensive approach. Although, MS/MS experiments allow for the enhancement of sensitivity by applying the selected reaction monitoring (SRM) mode for the determination of selected compounds, the high-resolution mass spectrometry (HRMS) approach enables the specific identification of analytes from the full scan data, making every measurement accessible to subsequent analysis and the search for new, previously not encountered compounds.

Therefore, to determine the presence of adulterants in WPFS, a liquid chromatography method coupled to Orbitrap high resolution mass spectrometry (LC-Orbitrap-HRMS) after solid phase extraction, was optimized and validated to detect the following different classes of substances: anabolic agents, beta-agonists, hormone and metabolic modulators, diuretics and stimulants.

2 Experimental

2.1 Quality assurance

All analytical and managerial procedures were carried out in an ISO/IEC 17025 standard environment, accredited by the Brazilian National Metrological Institute (BNMI - INMETRO) (Associação Brasileira de Normas Técnicas, 2005).

2.2 Chemicals and materials

All solvents used were HPLC grade: methanol, formic acid, ammonium formate and acetic acid (Tedia; Fairfield, USA), and the distilled water was purified by the milli-Q purification system (Millipore, Massachusetts, USA). Reference compounds were purchased mainly from NMI (Sydney, Australia), Sigma-Aldrich (St. Louis, USA) and Logical (Luckenwalde, Germany) or were kindly donated by other anti-doping laboratories. The Strata-X-CW, weak cation mixed mode polymeric sorbent (30 mg, 3 mL) SPE cartridges (São Paulo, Brazil) were purchased from Phenomenex. The internal standards mefruside, methyltestosterone, n-methylhexanamine, buspirone hydrochloride, 4-methylefedrine-D3 HCl, and 7-propyltheophylline were purchased from Sigma-Aldrich (St. Louis, USA).

2.3 Standard solutions

The fortification solution containing the reference standards consisted of a mixture of the following classes of substances: anabolic agents, beta-agonists, hormone and metabolic modulators, diuretics and masking agents and stimulants, at different concentrations (2 to 8 ng μL^{-1}), prepared in methanol.

The internal standard (IS) solution consisted of a mixture of the substances mefruside, methyltestosterone, n-methylhexanamine, propyl-theophylline and 7-propyl-theophylline at different concentrations (6 to 20 ng μL^{-1}), all dissolved in methanol.

2.4 Sample preparation

Eleven brands of WPFS were purchased from local Brazilian markets and 20 mg of each sample dissolved in 2 mL of water. Three controls were prepared: reagent blank (water), negative control (matrix without the analytes) and positive control (matrix spiked with 50 µL of fortification solution). The samples and controls were homogenized during 20 s and centrifuged for 20 min at 1.5 x G. One aliquot of the 10 µL in each test tube was transferred to a conical Eppendorf tube and 50 µL of the 2% (v/v) acetic acid solution added. The tubes were centrifuged for 5 min at 1.5 x G and stored at 2 °C to 6 °C for the subsequent reconstitution of the extract. The second aliquot taken from the supernatant obtained from each initial sample was transferred to a new test tube, 20 µL of the internal standard solution added and the contents homogenized by vortex for 20 s. In sequence, the solid-phase extraction (SPE) step was carried out. The SPE cartridges were conditioned with 1 mL of methanol and 1 mL of Milli-Q water; the sample was applied and the cartridges washed with 1 mL of Milli-Q water and 1 mL of 50% (v/v) methanol in water. The analytes were eluted with 1 mL of 5% (v/v) formic acid in methanol. All samples were evaporated under a nitrogen flow at 45 °C. The first aliquot was added to the dried residue and the mixture homogenized by vortex for 20 s, transferred to vials with the inserts and refrigerated at 4 °C for 4 h. The supernatants were transferred to new vials with inserts and injected into the chromatographic system.

2.5 Instrumentation

The liquid chromatography system was an Accela LC liquid chromatography (Thermo Scientific, Bremen, Germany), with an Accela 1250 pump and auto sampler fixed at 10 °C. The column was a Zorbax SB-C_{18} one, 3.0 mm × 50 mm, 1.8 µm (Agilent, Böblingen, Germany). The mobile phases were 0.1% ammonium formate/0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient program was as follows: 95% A for 0.5 min, then decreasing linearly to 90% at 10.0 min, then to 0% at 11.1 min, followed by an increase to the initial concentration of 95% A at 14.0 min. The total run time was thus 14 min. The column was maintained at 40 °C, the flow rate constant at 600 µL min^{-1} and the injection volume was 0.5 µL.

The LC effluent was pumped to a Q Exactive Orbitrap-based high resolution mass spectrometer (Thermo Scientific, Bremen Germany) operating in the positive-negative polarity switching mode and equipped with an electrospray ionization (ESI) source. The nitrogen gas flow and auxiliary gas were set to 60 and 20 (arbitrary units), respectively. The capillary temperature was 380 °C, the spray voltage 3900 kV - 2900 kV and the capillary voltage 3.9 V or -2.9 V, in the positive or negative modes, respectively. The instrument was operated in the full scan mode from *m/z* 100 to 620 and from 70 to 630, in the positive and negative modes, respectively, at 70,000 resolution power. The automatic gain control (AGC) was 10^6. The performance of the Orbitrap in both the positive and negative ionization modes was evaluated daily.

The data obtained after the LC-Orbitrap-HRMS analyses were processed using the Qual Browser (Thermo Electron, San Jose, CA), applying a 5 ppm tolerance error and FULL-MS acquisition. In addition, the formula calculator used included carbon, hydrogen and oxygen atoms to provide chemical formula and saturation

values (ring double-bonds equivalent - RDBE) for the precursor ions $[M+H]^+$ and $[M+H]^-$. A comparison between the theoretical and experimental precursor molecular mass values was evaluated in the identification of molecule structures. The instrumental conditions used were established according to anti-doping control (Sardela et al., 2018).

2.6 Method validation

The method was validated by qualitative screening, according to the internal validation protocol of the *Laboratório Brasileiro de Controle de Dopagem*, based on the DOC-CGCRE 008 protocol of the *Instituto Nacional de Metrologia, Qualidade e Tecnologia*. The selectivity evaluated ten different WPFS samples, declared as negative, and verified the absence of interfering substances in the retention times of each analyte. The limit of detection was evaluated by preparing 10 different samples spiked with 10% of the usual concentration and 10 different samples spiked with 50% of the usual concentration. The repeatability was determined using 7 replicates of WPFS, and the peak area values were expressed according to the relative standard deviation of each substance monitored. The recovery was evaluated by preparing 7 replicates spiked before the solid phase extraction step (repeatability) and 7 replicates spiked after the solid phase extraction step. The carryover was verified by an analysis of the spiked WPFS sample with twice the fortification solution concentration between 2 blank WPFS samples. The matrix effect evaluated 10 different WPFS samples, declared negative, which were spiked with the monitored analytes.

2.7. Application to real samples

The optimized and validated method by LC-HRMS was applied to the analysis of eleven commercial samples of WPFS products.

3 Results and discussion

3.1 Sample preparation

The WPFS samples contain a high concentration of the proteins β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins and glycomacropeptides, as well as other minor proteins such as lactoperoxidase, lactoferrin, β-microglobulin, lysozyme, insulin-like growth factor and others (Haraguchi et al., 2006). These proteins should be removed before injecting the sample into the chromatographic system. The presence of those compounds may clog the SPE column or the analytical column, due to precipitation, reducing the life of the column and competing for the electrospray ionization source, thus interfering in substance detection. Hence, it is indispensable to carry out sample pre-treatment procedures to remove these proteins, without loss of the target analytes. Thus a solvent precipitation step before the solid phase extraction was tested with different solvents, in order to reduce the amount of protein in the matrix, but not remove the target substances. Cold water, acetonitrile and a combination of acetonitrile/water (50% - v/v) were tested and the final recoveries of the substances compared based on their peak areas.

Acetonitrile and acetonitrile/water were not effective, because they also removed some target substances, especially the diuretics. Only the use of the cold water dissolved the WPFS and, at the same time, eliminated the excess of proteins and allowed for the detection of all the target substances with good recovery.

3.2 Method validation

Selectivity verifies the absence of interference in the retention times of the monitored substances. No significant interference in the retention times was observed for the target substances.

Table 1. Chemical formula, polarity, retention times (t_R), theoretical masses (*m/z*), sample concentration (SC), repeatability, recovery, limit of detection (LOD) and matrix effect of the monitored compounds.

Compound	Chemical Formula	Polarity	t_R (min)	*m/z*	SC	Repeatability	Extraction	LOD	Matrix
					(ng g^{-1})	(%)	yield (%)	(ng.g^{-1})	interference (%)
Andarine	$C_{19}H_{18}F_3N_3O_6$	-	6.07	440.10749	12.5	14.34	99.0	1.25	0.30
Gestrinone	$C_{21}H_{24}O_2$	+	7.00	309.18491	12.5	18.00	96.8	1.25	0.15
Methyldienolone	$C_{19}H_{26}O_2$	+	6.96	287.20056	12.5	10.06	95.8	1.25	0.11
Methyltrienolone	$C_{19}H_{24}O_2$	+	6.90	285.18491	12.5	6.87	95.1	1.25	0.14
Ostarine	$C_{19}H_{14}F_3N_3O_3$	-	6.46	388.09145	12.5	21.58	96.2	1.25	0.12
Oxandrolone	$C_{19}H_{30}O_3$	+	6.60	307.22677	12.5	8.26	95.7	1.25	0.13
Tetrahydrogestrinone	$C_{21}H_{28}O_2$	+	7.87	313.21621	12.5	18.49	96.3	1.25	0.14
Bamethan	$C_{12}H_{19}NO_2$	+	1.80	210.14886	50.0	1.94	91.6	5.00	1.17
Formoterol	$C_{19}H_{24}N_2O_4$	+	3.29	345.18088	100.0	5.38	92.0	10.00	1.12
Isoxsuprine	$C_{18}H_{23}NO_3$	+	3.71	302.17507	50.0	5.69	94.1	5.00	0.90
Metaproterenol	$C_{11}H_{17}NO_3$	+	0.49	212.12812	50.0	15.86	96.7	5.00	2.63
Procaterol	$C_{16}H_{22}N_2O_3$	+	1.96	291.17032	50.0	8.86	95.2	5.00	1.17
Ritodrine	$C_{17}H_{21}NO_3$	+	2.02	288.15942	50.0	5.53	97.8	5.00	1.24
Salmeterol	$C_{25}H_{37}NO_4$	+	6.33	416.27954	50.0	10.16	90.0	5.00	0.12
Aminoglutethimide	$C_{10}H_{12}N$	+	2.31	146.09642	50.0	10.80	92.8	5.00	0.94
Anastrazole	$C_{17}H_{19}N_5$	+	4.80	294.17132	50.0	7.44	92.8	5.00	0.14
Androstatrienedione	$C_{19}H_{24}O_2$	+	6.20	283.16926	50.0	9.49	95.7	5.00	0.13
Exemestane	$C_{20}H_{24}O_2$	+	6.77	297.18490	50.0	13.45	98.5	5.00	0.12
Flutamide	$C_{11}H_{11}F_3N_2O_3$	-	6.39	275.06381	50.0	13.42	97.4	5.00	0.29
Fulvestrant	$C_{32}H_{47}F_5O_3S$	+	8.79	607.32388	50.0	32.86	75.4	5.00	0.08
Gw501516	$C_{21}H_{18}F_3NO_3S_2$	+	8.70	454.07530	50.0	37.94	75.3	5.00	0.10
Raloxifene	$C_{28}H_{27}NO_4S$	+	5.00	474.17336	50.0	17.68	89.6	5.00	0.43
Bendroflumethiazide	$C_{15}H_{14}F_3N_3O_4S_2$	-	4.85	420.03051	125.0	10.21	92.7	12.50	0.26
Benzbromarone	$C_{17}H_{12}Br_2O_3$	+	8.48	422.92260	125.0	25.83	77.4	62.50	0.14
Benzthiazide	$C_{15}H_{14}ClN_3O_4S_3$	-	4.75	429.97622	62.5	10.73	94.9	6.25	0.11
Bumetanide	$C_{17}H_{20}N_2O_5S$	-	6.26	363.10202	125.0	34.93	29.5	62.50	0.13
Chlorothiazide	$C_7H_8ClN_3O_4S_2$	-	0.95	293.94155	125.0	22.76	5.6	12.50	1.21
Chlorthalidone	$C_{14}H_{11}ClN_2O_4S$	-	3.61	337.00553	125.0	13.94	9.8	62.50	0.34

Table 1. Continued...

Compound	Chemical Formula	Polarity	t_R (min)	*m/z*	SC (ng g^{-1})	Repeatability (%)	Extraction yield (%)	LOD (ng.g^{-1})	Matrix interference (%)
Clopamide	$C_{14}H_{20}ClN_3O_3S$	-	4.10	344.08411	62.5	12.04	40.8	6.25	0.10
Conivaptan	$C_{32}H_{26}N_4O_2$	+	5.84	499.21285	125.0	12.49	88.7	12.50	0.21
Cyclopenthiazide	$C_{13}H_{18}ClN_3O_4S_2$	-	5.30	378.03545	125.0	11.98	97.1	12.50	0.13
Cyclothiazide	$C_4H_{16}ClN_3O_4S_2$	-	4.88	388.01979	125.0	11.34	95.4	12.50	0.25
Diclofenamide	$C_6H_6Cl_2N_2O_4S_2$	-	2.52	302.90733	125.0	16.94	6.8	12.50	0.51
Etacrynic acid	$C_{13}H_{12}Cl_2O_4$	-	6.47	301.00399	125.0	35.81	15.3	12.50	0.38
Hydrochlorothiazide	$C_7H_8ClN_3O_4S_2$	-	1.18	295.95720	250.0	26.58	5.3	25.00	0.00
Hydroflumethiazide	$C_8H_8F_3N_3O_4S_2$	-	1.75	329.98356	62.5	24.89	4.1	6.25	0.24
Lixivaptan	$C_{27}H_{21}ClFN_3O_2$	+	7.52	474.13791	125.0	38.07	81.4	62.50	0.11
Methazolamide	$C_5H_8N_4O_3S_2$	-	2.12	234.99650	125.0	31.49	4.4	12.50	0.24
Methyclothiazide	$C_9H_{11}Cl_2N_3O_4S_2$	+	3.37	359.96408	500.0	5.36	117.7	250.00	0.38
Piretanide	$C_{17}H_{18}N_2O_5S$	-	5.84	361.08637	125.0	30.09	12.4	62.50	0.31
Polythiazide	$C_{11}H_{13}ClF_3N_3O_4S_3$	-	4.81	437.96360	62.5	8.62	95.9	6.25	0.14
Probenecid	$C_{13}H_{19}NO_4S$	-	6.24	284.09620	62.5	23.93	27.1	6.25	0.13
Spironolactone	$C_{24}H_{32}O_4S$	+	6.80	341.21112	62.5	13.94	99.3	6.25	0.17
Torasemide	$C_{16}H_{20}N_4O_3S$	-	4.77	347.11833	62.5	8.25	89.4	6.25	0.23
Triamterene	$C_{12}H_{11}N_7$	+	2.96	254.11487	62.5	5.24	94.2	6.25	1.49
Trichlormethiazide	$C_8H_8Cl_3N_3O_4S_2$	-	3.06	377.89490	125.0	16.54	21.5	12.50	0.44
Xipamide	$C_{15}H_{15}ClN_2O_4S$	-	5.61	353.03683	62.5	19.16	24.6	6.25	0.15
(s)-2-aminooctane	$C_8H_{19}N$	+	3.90	130.15902	125.0	2.94	91.5	12.50	1.19
3,3-diphenylpropylamine	$C_{15}H_{17}N$	+	4.68	212.14338	125.0	3.53	90.1	12.50	0.69
4-fluoroamphetamine	$C_9H_{12}FN$	+	2.00	154.10265	125.0	4.50	89.1	12.50	1.10
Amiphenazole	$C_9H_9N_3S$	+	1.59	192.05899	125.0	14.42	101.8	62.50	1.37
Benfluorex	$C_{19}H_{20}F_3NO_2$	+	5.80	352.15189	125.0	9.96	86.5	12.50	0.12
Benzphetamine	$C_{17}H_{21}N$	+	4.18	240.17468	125.0	4.24	91.2	12.50	0.83
Benzylpiperazine	$C_{11}H_{16}N_2$	+	0.90	177.13863	31.25	11.67	87.9	3.16	5.66
Carphedon	$C_{12}H_{14}N_2O_2$	+	3.18	219.11280	125.0	12.71	36.1	12.50	0.13
Cathine	$C_9H_{13}NO$	+	1.36	134.09640	250.0	5.69	88.3	25.00	1.70
Chlorphentermine	$C_{10}H_{14}ClN$	+	3.62	184.08875	125.0	3.76	91.3	12.50	1.45
Clobenxorex	$C_{16}H_{18}ClN$	+	4.93	260.12005	62.5	4.55	92.1	6.25	0.57
Cocaine	$C_{17}H_{21}NO_4$	+	3.24	304.15433	125.0	4.45	96.2	12.50	1.26

Table 1. Continued...

Compound	Chemical Formula	Polarity	t_R (min)	*m/z*	SC (ng g^{-1})	Repeatability (%)	Extraction yield (%)	LOD (ng.g^{-1})	Matrix interference (%)
Cropropamide	$C_{13}H_{24}N_2O_2$	+	5.10	241.19105	125.0	5.10	78.0	12.50	0.13
Crotethamide	$C_{12}H_{22}N_2O_2$	+	4.25	227.17540	125.0	15.34	70.6	12.50	2.00
Cyclazodone	$C_{12}H_{12}N_2O_2$	+	4.04	217.09715	125.0	7.37	87.3	12.50	0.25
Dobutamine	$C_{18}H_{23}NO_3$	+	2.73	302.17507	125.0	7.38	95.6	12.50	1.56
Etamivan	$C_{12}H_{17}NO_3$	+	4.16	224.12812	62.5	11.03	52.6	6.25	0.20
Etilefrine	$C_{10}H_{15}NO_2$	+	0.55	182.11756	125.0	7.64	96.6	12.50	3.12
Famprofazone	$C_{24}H_{31}N_3O$	+	6.40	378.25399	62.5	6.85	88.5	6.25	0.28
Fenbrutazate	$C_{23}H_{29}NO_3$	+	6.30	368.22202	62.5	8.59	87.8	6.25	0.24
Fencamine	$C_{20}H_{28}N_6O_2$	+	3.26	385.23465	62.5	7.79	92.9	6.25	0.89
Fenethyline	$C_{18}H_{23}N_5O_2$	+	3.53	342.19245	125.0	3.43	95.3	12.50	0.82
Fenfluramine	$C_{12}H_{16}F_3N$	+	3.93	232.13076	125.0	3.25	90.4	12.50	1.19
Fenproporex	$C_{12}H_{16}N_2$	+	1.92	189.13863	62.5	1.72	89.8	6.25	1.13
Flephedrone	$C_{10}H_{12}FNO$	+	4.33	182.09757	125.0	23.19	108.7	12.50	1.58
Furfenorex	$C_{15}H_{19}NO$	+	3.46	230.15394	125.0	4.24	88.8	12.50	1.17
Heptaminol	$C_8H_{19}NO$	+	1.30	146.15394	250.0	6.18	87.6	25.00	2.00
Isometheptene	$C_{24}H_{48}N_2O_8$	+	3.02	142.15903	125.0	2.95	90.4	12.50	1.55
Mefenorex	$C_{12}H_{18}CIN$	+	3.37	212.12005	62.5	2.62	90.3	6.25	1.40
Mephedrone	$C_{11}H_{15}NO$	+	2.50	178.12264	125.0	3.24	92.9	12.50	1.46
Mesocarb	$C_{18}H_{18}N_4O_2$	+	6.20	323.15025	125.0	14.89	93.0	12.50	0.13
Methoxyphenamine	$C_{11}H_{17}NO$	+	2.77	180.13829	62.5	3.15	92.6	6.25	1.48
Methylenodioxyamfetamine	$C_{10}H_{13}NO_2$	+	2.09	163.07540	125.0	5.92	38.8	12.50	1.23
Methylenodioxymethamfetamine	$C_{11}H_{15}NO_2$	+	2.14	194.11756	62.5	4.69	91.1	6.25	1.62
Methylenodioxy-n-ethylamfetamine	$C_{12}H_{17}NO_2$	+	2.46	208.13321	125.0	2.87	92.9	12.50	1.47
Methylephedrine	$C_{11}H_{17}NO$	+	1.61	180.13829	125.0	3.68	94.4	12.50	1.30
Methylphenidate	$C_{14}H_{19}NO_2$	+	3.28	234.14885	125.0	3.62	92.7	12.50	1.12
Mitragyne	$C_{23}H_{30}N_2O_4$	+	4.75	399.22783	125.0	6.59	91.7	12.50	0.41
Modafinil	$C_{15}H_{15}NO_2S$	+	4.85	296.07157	125.0	4.87	92.9	12.50	0.14
Nikethamine	$C_{10}H_{14}N_2O$	+	2.84	179.11789	62.5	15.14	8.3	6.25	0.00
Norfenfluramine	$C_{10}H_{12}F_3N$	+	3.60	204.09946	125.0	3.05	91.5	12.50	1.47
Octhylamine	$C_8H_{18}N$	+	4.35	130.15903	125.0	2.91	88.8	12.50	0.98
Oxilofrine	$C_{10}H_{15}NO_2$	+	0.39	133.06479	125.0	7.63	94.8	12.50	3.80

Table 1. Continued...

Compound	Chemical Formula	Polarity	t_R (min)	*m/z*	SC (ng g^{-1})	Repeatability (%)	Extraction yield (%)	LOD (ng.g^{-1})	Matrix interference (%)
Pemoline	$C_9H_8N_2O$	+	2.09	177.06585	125.0	32.90	4.7	12.50	0.25
Pentetrazol	$C_6H_{10}N_4$	+	2.03	139.09782	125.0	23.38	6.2	12.50	0.25
Phendimetrazine	$C_{12}H_{17}NO$	+	1.88	192.13829	125.0	2.68	91.3	12.50	3.65
Phenmetrazinha	$C_{11}H_{16}CINO$	+	1.92	178.12264	125.0	3.20	91.4	12.50	1.73
Pholedrine	$C_{10}H_{15}NO$	+	0.89	166.12264	125.0	9.77	94.6	12.50	4.30
p-hydroxy amphetamine	$C_9H_{13}NO$	+	0.85	135.08044	125.0	8.73	99.4	12.50	4.30
Pipradol	$C_{18}H_{21}NO$	+	4.07	268.16959	125.0	3.06	93.5	12.50	1.08
Prenilamyne	$C_{24}H_{27}N$	+	6.52	330.22163	125.0	11.48	90.2	12.50	0.12
Prolintane	$C_{15}H_{23}N$	+	3.82	218.19033	125.0	2.18	91.5	12.50	0.98
Propylhexedrine	$C_{10}H_{21}N$	+	3.79	156.17468	125.0	2.69	93.0	12.50	1.21
s(+)-methamphetamine	$C_{10}H_{15}N$	+	2.05	150.12773	62.5	3.90	91.5	6.25	1.42
Selegine	$C_{13}H_{17}N$	+	2.54	188.14338	125.0	5.28	88.2	12.50	1.24
Sibutramine	$C_{17}H_{26}CIN$	+	6.06	280.18265	125.0	7.17	86.4	12.50	0.13
Strychnine	$C_{21}H_{22}N_2O_2$	+	2.53	335.17540	125.0	3.97	90.4	12.50	1.29
Trimetazidine	$C_{14}H_{22}N_2O_3$	+	1.86	267.17032	125.0	7.17	82.2	12.50	1.20

Table 1 summarizes the results observed for all the substances validated. The detection limit (LOD) represents the lowest concentration of the substance that is detectable but not necessarily quantified using an experimental procedure. The LOD for anabolic agents was 1.25 ng g^{-1}, beta-agonists from 5 to 10 ng g^{-1}, hormone and metabolic modulators from 5 to 12.5 ng g^{-1}, diuretics from 6.25 to 62.5 ng g^{-1}, and stimulants and anorectic agents from 3.16 to 25 ng g^{-1}. All compounds were detected with more than 8 point-acquisitions at the LOD. This low limit of detection is associated with the screening method sensitivity and it is important for the screening of adulterants in commercial WPFS samples.

The repeatability was verified using the peak areas of each analyte from 7 WPFS replicates and Table 1 shows this variation as the relative standard deviation (RSD). The RSD values obtained were compared with the maximum relative standard deviation for each level of concentration calculated by the Horwitz equation (Horwitz et al., 1980). According to the comparison between the RSD and the values obtained using the Horwitz equation, the majority of the substances monitored (96.19%) showed an adequate relative standard deviation, indicating a low result dispersion. Only ethacrynic acid, benzbromarone, bumetanide and fulvestrant showed RSD values above the values preconized by the Horwitz equation.

The recovery can also be observed in Table 1 and it was lower than 50% for 15% of the targeted substances including ethacrynic acid, benzbromarone, bumetanide and fulvestrant again, and also clopamide, chlorothiazide, chlorthalidone, diclofenamide, hydrochlorothiazide, hydroflumethiazide, methazolamide, piretanide, probenecid, carphedon, methylenedioxyamfetamine, nikethamide, pemoline, pentetrazol, trichlormethiazide and xipamide. Almost all of these substances are acids. However, despite the recovery below 50%, all the substances were properly detected in all the replicates, mainly because by HRMS, low noise and clear signals were obtained, allowing the presence of the substances to be detected.

As a consequence of this comprehensive sample preparation procedure, a high influence of the matrix in the instrumental conditions was expected. When complex matrices such as WPFS are analyzed, signal suppression of an analyte can occur and/or a shift in the retention time can be observed, probably because of the punctual modification of the stationary phase or due to overlay of the WPFS matrix components. The ion suppression was evaluated in the matrix effect experiments and the variation in the retention time (t_R) was evaluated by monitoring the t_R peak for all targeted substances for their respective retention times in a window of 1 minute. After injecting 10 different replicates, the highest RSD observed was 2% for the t_R of the substances that elute before 1 minute (Table 1).

Finally, the existence of carryover was tested, but none was observed. Carryover verifies the existence of significant variations amongst sequential injections.

3.3 Application to the commercial samples

The eleven brands of WPFS (identified by the numbers 1 to 11) were analyzed and compared with the positive control to check for the presence of adulterants. According to the LC-Orbitrap-HRMS analysis, peaks with retention times and *m/z* ratios equal or similar (error below 5 ppm) to the substances conivaptan, polythiazide and benfluorex, were found in some commercial samples. The samples showing a suspicion of adulteration were extracted and analysed twice more to confirm the presence of the adulterants.

Suspicious samples were confirmed based mainly on the parameters of the mass/charge ratios of the precursor ions, mass accuracy calculation, the RDBE values of the samples, and comparison with the positive control. Variations in the t_R and *m/z* ratios of the positive control and the commercial samples were observed in the suspect peaks. Subsequently the mass accuracy was calculated (maximum limit of 5 ppm for confirmation of the identities of the compounds) and the RDBE values. Figure 1 shows the retention times (t_R, min), molecular ions, calculation of mass accuracy, and the RDBE values relevant to the presence of the following diuretics: conivaptan, polythiazide and/or the stimulant: benfluorex.

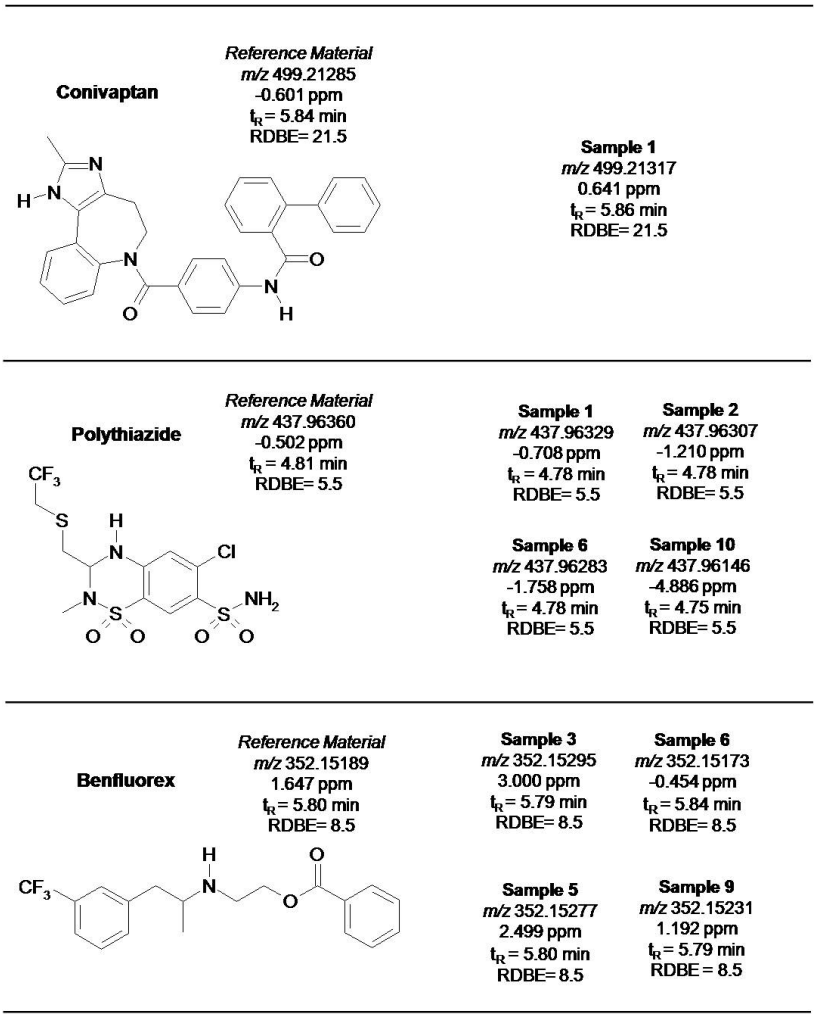

Figure 1. Chemical structures, *m/z*, ppm error, retention time and RDBE of conivaptan, polythiazide and benfluorex in the reference material and in the samples.

According to the comparison of the parameters, the values obtained for the samples suspected of adulteration were compatible with those of the positive control, confirming the presence of adulterants in those whey protein food supplements. After applying the method, seven of the eleven brands analysed (63.64%) showed adulteration by at least one of the above-mentioned substances.

The administration of these compounds can cause health risks (depending on the associated factors) and another aggravating fact is that many individuals are consuming products classified as foods without knowing that they may contain substances with pharmacological properties. The adulteration by diuretics and/or anorectic stimulants is related to the effects they may cause, and an effective weight loss is amongst the effects common to these classes. Conivaptan and polythiazide act by increasing diuresis, masking the other substances present, and benfluorex is a stimulant with an anorectic effect that induces a loss of appetite (Docherty, 2008; Woo et al., 2013).

4 Conclusions

Whey protein food supplement samples contain a high concentration of proteins which can be removed by solvent precipitation and solid-phase extraction clean-up before sample injection into the chromatographic system. Cold water was the best solvent option to remove these proteins and maintain the procedure comprehensive.

The LC-Orbitrap-HRMS method allowed for better separation, detection and identification of the analytes, due to the high sensitivity and resolution of the mass analyser.

The parameters selectivity, LOD, repeatability, extraction yield, carryover and matrix effect were duly validated. All these parameters showed satisfactory results in the detection of the substances with pharmacological action in the WPFS matrix.

After applying the method, it was shown that four commercial samples were not adulterated and seven were. One showed the presence of both conivaptan and politiazide (a combination of diuretic agents), two showed the presence of politiazide and benfluorex, and the others only showed the presence of either politiazide or benfluorex.

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