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DETECTION OF DIURETICS IN WHEY PROTEIN UTILIZING LIQUID-LIQUID EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LLE-LC-MS/MS): A COMPREHENSIVE ANALYSIS

(Pengesanan Diuretik dalam Protin Whey Menggunakan Pengekstrakan Cecair-Cecair Berpasangan dengan Kromatografi Cecair - Spektrometri Jisim (LLE-LC-MS/MS): Satu Analisis Komprehensif)

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Abstract

Protein supplements are widely consumed by athletes and recreationally active adults to enhance training adaptation, performance, and muscle recovery. However, the popularity of these supplements has led to instances of adulteration with banned substances, including diuretics. Diuretics, typically used for medical conditions like hypertension and edema, are illicitly added to supplements to promote weight loss and mask other doping agents. Their presence poses significant health risks, including electrolyte imbalances and renal dysfunction, and contributes to doping violations in sports. Despite the critical need to monitor diuretic contamination in protein supplements, there is a noticeable gap in the literature regarding optimized extraction methods for these compounds. This study aims to evaluate the efficiency of various solvents in extracting diuretic compounds from protein supplements. By assessing the extraction recovery rates and optimizing the selection of solvents extraction, this research seeks to enhance the sensitivity, specificity, and accuracy of liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. This study investigates the extraction efficiency and matrix effects of methanol and acetonitrile for detecting diuretics in whey protein using Liquid-Liquid Extraction (LLE) coupled with LC-MS/MS. Different solvent compositions 100%, 70%, and 50% methanol (100M, 70M, 50M) and acetonitrile (100A, 70A, 50A) were evaluated to optimize recovery rates and minimize matrix interference. Results show that methanol consistently outperformed acetonitrile in recovering diuretic compounds from whey protein. At 100M demonstrated the highest average recovery rates (49.639% to 99.735%) with moderate signal enhancement and minimal suppression effects, indicating effective mitigation of matrix interference. Similarly, 70M maintained balanced matrix effects and reliable recoveries (46.976% to 94.492%), making it a robust alternative for diuretic analysis. In contrast, acetonitrile exhibited greater variability in matrix effects and lower recovery rates. For instance, 100A showed significant signal suppression (0.070% to 9.267%), suggesting limitations in solubilizing diuretics from whey protein. While 70A provided a more stable profile, it still showed variability (matrix effects from -44.539% to 29.493%) compared to methanol. The study highlights the critical role of solvent selection in minimizing matrix effects and ensuring accurate diuretic quantification in complex food matrices. Methanol's superior solvating power and polarity contribute to its effectiveness in mitigating matrix interference compared to acetonitrile. This research provides valuable insights for analytical chemists and food scientists aiming to improve the accuracy and consistency of diuretic analysis in food matrices, thereby ensuring consumer safety and regulatory compliance.

Keywords: diuretics, liquid-liquid microextraction, liquid chromatography, whey protein

Abstrak

Suplemen protin digunakan secara meluas oleh atlit dan individu yang aktif secara rekreasi untuk meningkatkan penyesuaian latihan, prestasi, dan pemulihan otot. Namun, populariti suplemen ini telah menyebabkan terjadinya pemalsuan dengan bahan terlarang, termasuk diuretik. Diuretik, yang biasanya digunakan untuk keadaan kesihatan seperti hipertensi dan edema, telah ditambah ke dalam suplemen secara haram bagi menurunkan berat badan dan menyembunyikan agen doping yang lain. Kehadiran bahan-bahan tersebut menyebabkan risiko kesihatan yang jelas, termasuklah ketidakseimbangan elektrolit dan ketidakfungsian ginjal, serta menyumbang kepada pelanggaran doping dalam sukan. Meskipun pemantauan pencemaran diuretik di dalam suplemen protin diperlukan secara kritikal, terdapat jurang yang ketara di dalam literatur mengenai kaedah-kaedah pengekstrakan yang optimum untuk sebatian ini. Kajian ini bertujuan untuk menilai keberkesanan bagi pelbagai jenis pelarut dalam mengekstrak sebatian diuretik daripada suplemen protin. Dengan menilai kadar pemulihan pengekstrakan dan mengoptimum pemilihan pengekstrakan pelarut, kajian ini bertujuan untuk meningkatkan kepekaan, kekhususan, dan ketepatan bagi analisis kromatografi cecair-spektrometri jisim (LC-MS/MS). Kajian ini menyiasat keberkesanan pengekstrakan dan kesan matrik bagi metanol dan acetonitril untuk mengesan diuretik di dalam protin whey menggunakan pengekstrakan cecair-cecair (LLE) berpasangan dengan LC-MS/MS. Komposisi pelarut yang berbeza anataranya 100%, 70%, dan 50% kepekatan metanol (100M, 70M, 50M) dan acetonitril (100A, 70A, 50A) telah dinilai untuk mengoptimum kadar pemulihan dan meminimumkan gangguan matrik. Hasil kajian menunjukkan bahawa metanol menunjukkan prestasi tinggi secara konsisten berbanding acetonitril dalam pengekstrakan sebatian diuretik daripada protin whey. Purata kadar pemulihan pada 100M menunjukkan yang tertinggi (49.639% to 99.735%) dengan kesan penambahan isyarat yang sederhana dan pengurangan isyarat yang minimum, ini menunjukkan keberkesanan dalam pengurangan gangguan matrik. Sama seperti 100M, 70M juga mengekalkan kesan matrik yang seimbang dan pemulihan yang boleh dipercayai (46.976% to 94.492%), menjadikan ia sebagai alternatif yang teguh bagi analisis diuretik. Manakala acetoniril pula menunjukkan kebolehubahan yang lebih besar dalam kesan matrik dan kadar pemulihan yang lebih rendah. Sebagai contoh, 100A menunjukkan pengurangan isyarat yang ketara (0.070% hingga 9.267%), ini menunjukkkan batasan dalam melarutkan diuretik daripada protin whey. Walaupun 70A memberikan profil yang lebih stabil, ia masih menunjukkan kebolehubahan (kesan matrik daripada -44.539% kepada 29.493%) berbanding metanol. Kajian ini menyerlahkan peranan penting pemilihan pelarut dalam meminimumkan kesan matrik dan memastikan kuantifikasi diuretik yang tepat dalam matriks makanan yang kompleks. Kuasa pelarut metanol yang unggul dan kepolarannya menyumbang kepada keberkesanannya dalam mengurangkan gangguan matriks berbanding dengan asetonitril. Kajian ini memberikan pandangan yang berharga untuk ahli kimia analisis dan saintis makanan yang bertujuan untuk meningkatkan ketepatan dan konsistensi analisis diuretik dalam matrik makanan, dengan itu memastikan keselamatan pengguna dan pematuhan peraturan.

Kata kunci: diuretik, pengekstrakan cecair-cecair, kromatografi cecair, protin whey

Introduction

Protein supplements are frequently consumed by athletes and recreationally active adults to enhance training adaptation and performance as well as to accelerate muscle recovery [1-3]. Because of the popularity of protein powders, it has been a of target adulteration with substitutes products such as banned substances and cheap proteins [4]. The main cases of sports supplement adulteration are related to the following classes of banned substances including anabolic agents, diuretics and stimulants [5, 6].

Diuretics are medications commonly used in the management of conditions such as liver cirrhosis, heart failure, hypertension, and edema. These drugs work by increasing urine production, which leads to the removal of excess fluid and electrolytes from the body [7] therefore, they are illegally added to dietary supplements used for effective weight loss [8]. However, their misuse or undisclosed presence in dietary supplements can lead to adverse effects such as electrolyte imbalances, renal dysfunction, and increased risk of conditions like Alzheimer's disease and gout [9, 10]. Beside the health issue, taking diuretics also can tend to doping issue among athletes [11]. Diuretics were first banned in sport in 1988 because they can be used by athletes for two primary reasons. First, their potent ability to remove water from the body can cause a rapid weight loss that can be required to meet a weight category in sporting events. Second, they can be used to mask the administration of other doping agents by reducing their concentration in urine primarily because of an increase in urine volume. The urine dilution effect of diuretics also allows them to be classified as masking agents and precludes their use both in and out of competition. Some diuretics also cause a masking effect by altering the urinary pH and inhibiting the passive excretion of acidic and basic drugs in urine [11-13]. Therefore, understanding the presence and concentration of diuretic compounds in protein supplements is crucial for regulatory bodies and athletes to ensure fair play, health safety, and adherence to anti-doping regulations.

Despite the significant health and doping issues of diuretic compounds in protein supplements, there is a noticeable gap in the existing literature concerning the extraction methods specifically tailored for these compounds. Many studies may have focused on the identification and quantification of diuretic compounds but might not have thoroughly explored the extraction process itself. The selection of suitable solvent for the extraction of diuretic compounds is a critical factor in the efficiency of extraction in liquid-liquid extraction (LLE) coupled with Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) studies. The selection of a solvent should prioritize minimal toxicity [14], while considering physical and chemical properties such as density and viscosity, which can influence the extraction process and compound solubility [15]. Numerous studies have highlighted the use of 100% methanol as an effective extraction solvent in drug analysis for dietary supplements. Notably, some researchers have been reported the successful extraction of diuretics using the LLE method with 100% methanol [6, 16-17]. They found that simple pre-treatment by dissolving samples in 100% methanol, followed by LC-MS/MS analysis, allowed for efficient screening and quantification of diuretics in dietary supplements. Additionally, Akamatsu & Mitsuhashi successfully utilized methanol: water (70:30) for extracting diuretics from dietary supplements [18]. On the other hand, there are fewer reports on using acetonitrile as an extraction solvent. However, publications by Sciex demonstrated the use of acetonitrile in solvent extraction, specifically acetonitrile:water:acetic acid (70:29:1 and 79:20:1) for mycotoxin extraction in food samples [19, 20]. These studies showed that the LLE method coupled with SCIEX LC-MS/MS provides high-quality quantitation of compounds.

The primary goals of this investigation are to thoroughly assess the effectiveness of solvents in extracting diuretic compounds found in protein supplements. This entails a methodical examination of solvent performance, considering the extraction recovery of diuretic components from the intricate matrices of protein supplements. Employing LC-MS/MS, the study seeks to attain heightened sensitivity, specificity, and accuracy in the identification and quantification of diuretic compounds. LC-MS/MS is chosen for this study due to its superior ability to provide high sensitivity and selectivity in detecting low concentrations of analytes in complex matrices [21-23], compared to other analytical techniques such as high-performance liquid chromatography (HPLC) [24, 25]. This advanced technique allows for the simultaneous separation and precise identification of diuretic compounds, ensuring comprehensive and reliable analysis. Through these analytical techniques, the research aims to establish a robust platform, ensuring a comprehensive understanding of the diuretic content present in sports supplements.

Materials and Methods

Chemicals

The whey protein powder sample was obtained from the Malaysia market. All the reference standards of diuretics were obtained from various pharmaceutical suppliers. Cyclothiazide ($C_{14}H_{16}ClN_3O_4S_2$), bumetanide ($C_{17}H_{20}N_2O_5S$), spironolactone ($C_{24}H_{32}O_4S$), ethacrynic acid ($C_{13}H_{12}C_{l2}O_4$), dichlorphenamide ($C_6H_6C_{l2}N_2O_4S_2$), methazolamide ($C_5H_8N_4O_3S_2$), hydroflumethiazide ($C_8H_8F_3N_3O_4S_2$), trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$), torsemide ($C_{16}H_{20}N_4O_3S$), methyclothiazide ($C_9H_{11}C_{l2}N_3O_4S_2$), metolazone ($C_{16}H_{16}ClN_3O_3S$), and furosemide ($C_{16}H_{16}ClN_3O_3S$) were purchased from United States Pharmacopeia (Rockville MD, USA). Bendroflumethiazide ($C_{15}H_{14}F_3N_3O_4S_2$), canrenone ($C_{22}H_{28}O_3$), piretanide ($C_{17}H_{18}N_2O_5S$), amiloride ($C_6H_8ClN_7O$) and chlortalidone ($C_{14}H_{11}ClN_2O_4S$) were purchased from European Pharmacopoeia (Strasbourg, France). 4-Chloro-3-sulfamoylbenzoic acid ($C_7H_6ClNO_4S$), chlorothiazide ($C_7H_6ClN_3O_4S_2$), indapamide ($C_{16}H_{16}ClN_3O_3S$), acetazolamide

($C_4H_6N_4O_3S_2$), triamterene ($C_{12}H_{11}N_7$) and hydrochlorothiazide ($C_7H_8ClN_3O_4S_2$) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ultrapure water (H_2O) was produced by a Purelab Flex 3, Elga Veolia (Woodridge, USA). All solvents used are LC/MS grade (99.9%) of acetonitrile (C_2H_3N) and methanol (CH_4O) from Fisher Chemical (Belgium, UK). Formic acid (C_3H_7NO) and acetic acid ($C_2H_4O_2$) also from Fisher Chemical (Belgium, UK).

Extraction process for recovery study and matrix effects

In this study, we employed pre-spike, post-spike, and neat blank sample preparations to validate the recovery of diuretics in whey protein samples. Whey protein samples were collected from Malaysia market. For pre-spike samples, we prepared a standard solution of the mixture of 23 diuretics at a known concentration (10 µg/mL) and spiked the whey protein to achieve a final concentration of 100 ng/mL. For post-spike samples, 1.0 g of un-spiked whey protein powder was extracted with 10.0 mL of extraction solvent (different ratio of methanol and acetonitrile) was added to 1.0 g of whey protein powder. The filtered extract was then spiked with 50 µL of diuretics standard solution to achieve a final concentration of 100 ng/mL. The spiked extract was mixed thoroughly and analyzed immediately to determine the recovery rate. Neat blank samples were prepared by processing additional the standard solution of diuretics without adding any whey protein samples, following the same extraction and filtration procedures, and analyzed to confirm the absence of matrix or interference.

All samples, including pre-spike, post-spike, and neat blanks, were added with 10.0 mL of extraction solvent (different ratio of methanol and acetonitrile) was added to 1.0 g of whey protein powders, followed by thorough mixing using an orbital shaker for 30 minutes and the samples were centrifuged (4000 rpm) for 20 min. The different ratio of the extraction solvent as prepared as acetonitrile: H_2O: acetic acid (100.0:0:0, 70:29:1 and 50:49:1) and methanol: H_2O: acetic acid (100:0:0, 70:29:1 and 50:49:1). The supernatant layer was collected and diluted with 1-fold dilution using a mixture 35% of acetonitrile in water with 0.1 % formic acid. Before being injected into the LCMS/MS system, the samples were filtered with a 0.22 µm of PTFE filter.

In analytical chemistry, evaluating the performance of an extraction method is critical to ensuring accurate quantification of target compounds. Two essential metrics for this evaluation are percentage recovery and percentage matrix effect. Percentage recovery assesses the accuracy of an analytical method by determining how much of the known spiked amount of analyte is recovered after the extraction process. This metric indicates the efficiency of the extraction procedure and is calculated using the following equation:

$$Percentage\ Recovery\ (\%) = \frac{Peak\ area\ of\ Pre-spike}{Average\ Peak\ Area\ of\ n\ Post-spike}\ X\ 100 \qquad (1)$$

The matrix effect evaluates the influence of other components in the sample on the analyte's signal during analysis. This is crucial as it affects the accuracy and reliability of the measurement. The percentage matrix effect is calculated as:

$$Matrix\ Effect\ (\%) = \left(1 - \frac{Peak\ Area\ of\ Post\text{-}spike}{Average\ Peak\ Area\ of\ n\ Neat\ Blank}\ x\ 100\right) \qquad (2)$$

Where n ≥ 3.

Instrumentation

The liquid chromatography (LC) analysis will be performed with an Exion LC, AB Sciex, MA, USA. The chromatographic separation will be carried out using a 100 mm x 2.1 mm x1.6 µm, ACE Excel 3 C18-PFP UHPLC column (Ace, Scotland) with oven temperature maintained at 40 °C. The injection volume is 5 µl. Mobile phases A and B are ultra-pure water and acetonitrile respectively, both containing 0.1 % formic acid. The gradient in positive mode was performed as follow: 0 - 1.5 min mobile phase B 20%, 1.5 - 9 min mobile phase B 20 - 60%, 9 - 16 min mobile phase B 60 - 95%, 16 -16.1 min mobile phase B 95 - 20%, 16.1- 18 min mobile phase B 20%. In negative mode was performed as follow: 0 - 1 min mobile phase B 10%, 1 - 9 min mobile phase B 10 - 60%, 9 - 16 min mobile phase B 60 - 95%, 16 -16.1 min mobile phase B 95 - 10%, 16.1 - 18 min mobile phase B 10%. In both flow rate was 0.2 ml/min.

For mass spectrometry (MS) EPI analysis, an AB Sciex 4500 QTRAP system (MA, USA) equipped with electrospray ionization (ESI) source. The ion spray voltages were set to 5500 V in positive mode and 4500 V in negative mode. In both modes with the following parameter were set curtain gas: 25 psi; nebulizer gas: 40 psi; turbo gas: 60 psi; source temperature: 550 °C. Nitrogen served as nebulizer gas and collision gas. The survey scan was scheduled MRM with optimized declustering potential (DP), entrance potential (DP), entrance potential (EP) and collision energy (CE) was showed in Table1. All data were acquired using Analyst Software and processed by MultiQuant Software version 3.0.2.

Results and Discussion

Instrumentation Optimization

LCMS/MS is a widely used high-throughput technique. The multiple reaction monitoring (MRM) scanning capability offers the high sensitivity and selectivity to detect 23 of diuretic compounds. All the 23 diuretic compounds could be separated within 20 min using an ACE C18-PFP (100 mm x 2.1 mm x1.6 µm). To improve the peak shape of MS detection sensitivity and the separation efficiency, a gradient mode was applied with different starting gradient mode of the acetonitrile such as 10%, 20% and 30%, and the good separation of the analytes was obtained in 13 min. The best ratio of the starting gradient mode of the acetonitrile for positive and negative mode are 20% and 10%, respectively. Besides, to optimize the HPLC conditions 0.1% of formic acid was adopted as the mobile phase modifiers. These conditions gave good separation and high peak sensitivity of the compound's chromatogram. The flow rate was maintained to 0.2 ml/min, which is this flowrate suitable with the column.

Table 1 shows the data obtained for the screening of 23 of diuretic compounds. 12 substances (acetazolamide, amiloride, bendroflumethiazide, bumetanide, canrenone, indapamide, methazolamide, metolazone, piretanide, spironolactone, torsemide, triamterene**)** were ionized in positive ion mode and 11 substances (4-chloro-3-sulfamoylbenzoic acid, chlorothiazide, chlortalidone, ethacrynic acid, cyclothiazide, dichlorphenamide, furosemide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide and trichlormethiazide) were ionized in negative mode. The MRM1 and MRM2 of the protonated molecules ($[M+1]^+$) and their fragment ions of the acetazolamide (222.9/180.9, 222.9/163.8), amiloride (229.9/170.9, 229.9/115.9) bendroflumethiazide (421.9/91.0, 421.9/119.0), bumetanide (365.0/240.0, 365.0/184.0), canrenone (341.1/91.0, 341.1/107.0), indapamide (366.0/132.0, 366.0/91.0), methazolamide (236.9/115.9, 236.9/194.9), metolazone (365.9/178.9, 365.9/258.8), piretanide (363.0/236.0, 363.0/282.0), spironolactone(341.2/106.9, 341.2/187.3), torsemide (349.0/ 263.9, 349.0/168.0), triamterene (254.0/103.9, 254.0/237.0) as listed in Table 1. Meanwhile, the MRM1 and MRM2 of the deprotonated molecules ($[M-H]^-$) and their fragment ions of the 4-chloro-3-sulfamoylbenzoic acid (233.7/189.8, 233.7/79.8), chlorothiazide (293.7/213.8, 293.7/178.8), chlortalidone (337.1/146.1, 337.1/189.7), ethacrynic acid (302.9/256.8, 302.9/178.9), cyclothiazide (388.1/269.0, 388.1/204.8), dichlorphenamide (302.7/77.8, 302.7/238.7), furosemide (328.8/204.8, 328.8/284.8), hydrochlorothiazide (295.7/268.7, 295.7/204.8), hydroflumethiazide (329.8/238.8, 329.8/159.8), methyclothiazide (357.8/321.9, 357.8/257.9) and trichlormethiazide (379.6/241.7, 379.6/241.7) also listed in Table 1. The chromatogram in Figure 1 illustrates the profiles of 23 diuretic compounds based on their mode. In the positive mode, the retention time ranges from approximately 2 to 12 minutes, while in the negative mode, it ranges from around 6 to 12 minutes.

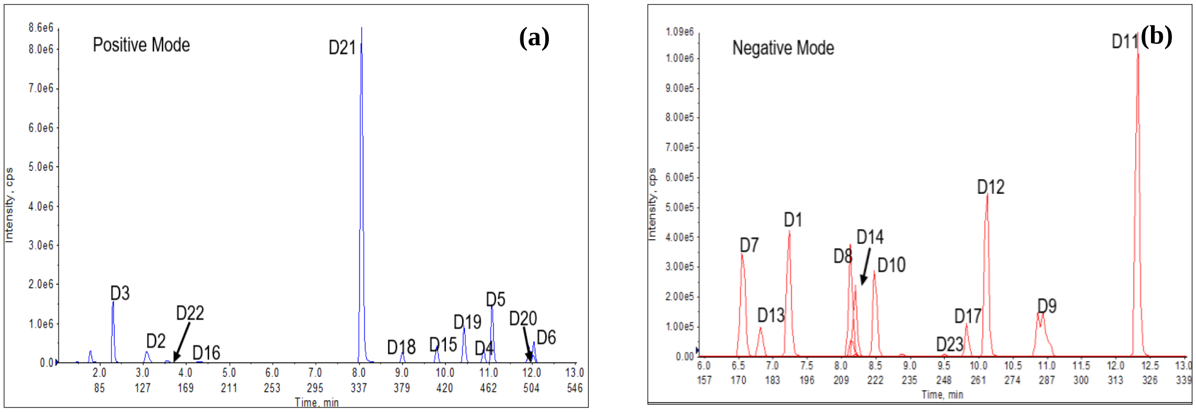

Figure 1. LCMS/MS Chromatogram of the 23 of diuretic compounds (a) positive mode and (b) negative mode

Table 1. MRM parameters and retention times (RT) for the diuretic compounds

Code	Name of Compound	ESI Polarity	RT (min)	MRM1	CE1 (volts)	MRM2	CE2 (Volts)
D1	4-Chloro-3-sulfamoylbenzoic acid	-	7.23	233.7/189.8	-16	233.7/79.8	-28
D2	Acetazolamide	+	3.08	222.9/180.9	19	222.9/163.8	27
D3	Amiloride	+	2.30	229.9/170.9	23	229.9/115.9	39
D4	Bendroflumethiazide	+	10.87	421.9/91.0	75	421.9/119.0	21
D5	Bumetanide	+	11.07	365.0/240.0	23	365.0/184.0	29
D6	Canrenone	+	12.04	341.1/91.0	77	341.1/107.0	31
D7	Chlorothiazide	-	6.56	293.7/213.8	-38	293.7/178.8	-54
D8	Chlortalidone	-	8.20	337.1/146.1	-24	337.1/189.7	-36
D9	Cyclothiazide	-	10.93	388.1/269.0	-36	388.1/204.8	-41
D10	Dichlorphenamide	-	8.48	302.7/77.8	-54	302.7/238.7	-15
D11	Ethacrynic acid	-	12.00	302.9/256.8	13	302.9/178.9	35
D12	Furosemide	-	10.30	328.8/204.8	-28	328.8/284.8	-20
D13	Hydrochlorothiazide	-	6.82	295.7/268.7	-24	295.7/204.8	-30
D14	Hydroflumethiazide	-	8.13	329.8/238.8	-32	329.8/159.8	-44
D15	Indapamide	+	9.76	366.0/132.0	17	366.0/91.0	55
D16	Methazolamide	+	4.30	236.9/115.9	35	236.9/194.9	19
D17	Methyclothiazide	-	9.82	357.8/321.9	-16	357.8/257.9	-22
D18	Metolazone	+	9.00	365.9/178.9	47	365.9/258.8	25
D19	Piretanide	+	10.42	363.0/236.0	39	363.0/282.0	27
D20	Spironolactone	+	12.03	341.2/106.9	32	341.2/187.3	30
D21	Torsemide	+	8.05	349.0/263.9	21	349.0/168.0	57
D22	Triamterene	+	3.56	254.0/103.9	33	254.0/237.0	9
D23	Trichlormethiazide	-	9.47	379.6/241.7	-40	379.6/241.7	-40

ESI: electrospray ionization, RT: retention time, MRM: multiple reaction monitoring, CE: collision energy

Comparison of solvent performance

In this study, various extraction methods were evaluated for their effectiveness in extracting diuretic compounds from whey protein powder using methanol and acetonitrile at different concentrations: 100%, 70%, and 50%. These methods were labeled as 100A, 70A, and 50A for acetonitrile, and 100M, 70M, and 50M for methanol. A 35% acetonitrile in water solution with 0.1% formic acid was used as a diluent to enhance separation and peak shape during analysis. The recovery efficiencies of diuretics under these conditions are presented in Figure 2 and Table 2. Based on Table 2, methanol generally exhibited higher average recovery rates across all concentrations tested. For instance, 100M had an average recovery range of approximately 49.63% to 99.73%, significantly higher than the 100A range of 0.07% to 9.26%. This indicates methanol's superior solubility and extraction capability for diuretics from the complex whey protein matrix [26].

Intermediate concentrations of methanol and acetonitrile (70M and 70A) also showed relatively high recoveries, ranging of average percentage recoveries from 46.97% to 94.49% for methanol and 48.54% to 93.96% for acetonitrile. Notably, 70% acetonitrile demonstrated high recovery rates, like 70% methanol, indicating that this concentration of acetonitrile and methanol can effectively balance solvent strength and selectivity, optimizing extraction while minimizing matrix interference. This balance enhances the solubility characteristics and diversity of recovered analytes. However, reducing the solvent concentration to 50% (50M and 50A) led to a decrease in the recovery rates of diuretics. This is attributed to the increased concentration of water, which decreases the recovery efficiency of diuretics. Previous research by Ji et al. [25] also demonstrated that adding water to methanol did not improve recovery rates of compounds.

Methanol, especially at 100% and 70%, demonstrated superior performance in extracting diuretics from whey protein, showing higher and more consistent recovery rates compared to acetonitrile. Several studies have highlighted methanol as an effective extraction solvent in drug analysis for dietary supplements using the LLE method [6, 16-18]. However, 70% acetonitrile also exhibited high recoveries, indicating its effectiveness under certain conditions. These findings are supported by publications from Sciex, which demonstrated the use of acetonitrile in solvent extraction, specifically acetonitrile:water:acetic acid (70:29:1), for extracting compounds in food samples, yielding high-quality extraction and quantitation results [20]. Therefore, the choice of solvent concentration is crucial, with 100% and 70% methanol and 70% acetonitrile providing the best results. Lower concentrations, such as 50%, were less effective due to increased water polarity and matrix interference. Optimizing solvent ratios is essential for improving the extraction efficiency of diuretics from complex matrices like whey protein.

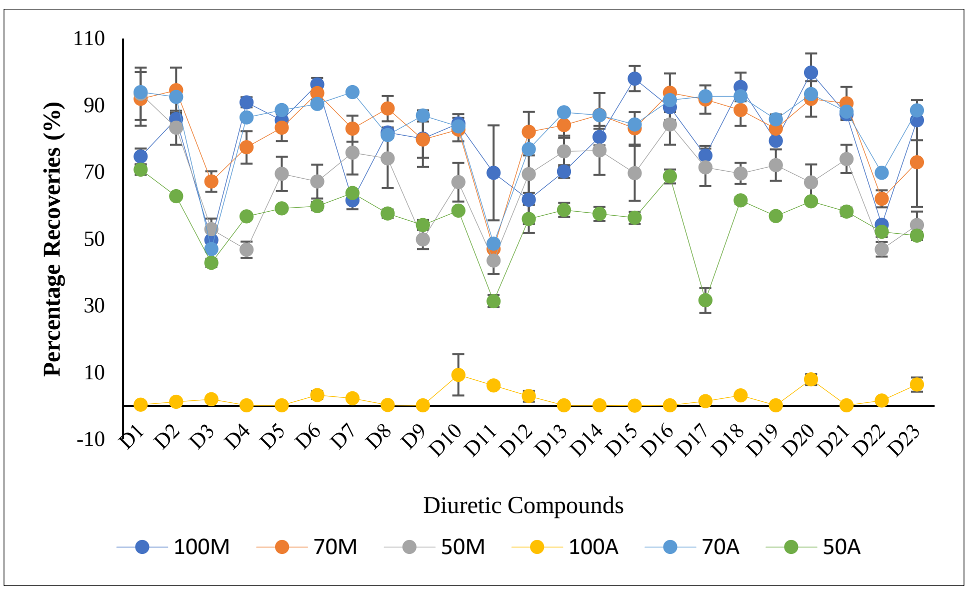

Figure 2. Percentage recoveries (%) of diuretic compounds using different types of solvent

Matrix effects analysis

Matrix effects are crucial in analytical chemistry as they can significantly impact the quantification and detection of analytes. In this study, methanol generally performed better than acetonitrile in minimizing matrix effects. The matrix effect percentages for diuretic compounds across different types and ratios of solvents are shown in Table 3. Methanol, particularly at 100M, showed the most consistent performance with moderate signal enhancement and fewer instances of significant suppression. The matrix effects for 100M ranged from -38.62% to 22.50%, indicating its superior ability to mitigate matrix interferences. This consistency suggests that 100M is effective in reducing the negative impact of matrix components on analyte detection. In contrast, acetonitrile demonstrated more variability in matrix effects. For instance, 100A exhibited a range of matrix effects from -89.83% to 27.43%. This significant signal suppression indicates that 100A is less effective in mitigating matrix interferences.

The 70M solution also demonstrated balanced matrix effects, making it a reliable alternative. The matrix effects for 70M ranged from -70.18% to 12.75%. Despite one extreme suppression value of -70.18% for chlorothiazide (D7) compound, most of the matrix effects were moderate, indicating that 70M strikes a good balance between solvent strength and matrix interference reduction. Among the acetonitrile concentrations, 70A provided a more stable profile with matrix effects ranging from -44.53% to 29.49%. Although it showed more variability than 70M, 70A had fewer extreme values, making it a better option among the acetonitrile concentrations tested. The high variability and extreme matrix effects observed with 50% methanol (50M) and 50% acetonitrile (50A) suggest that these solvent systems are less effective in controlling matrix interferences, leading to inconsistent recovery rates. For instance, 50M exhibited matrix effects ranging from -179.31% to 61.78%, indicating high variability and extreme values. Similarly, 50A showed matrix effects ranging from -171.02% to 68.65%, further highlighting the inconsistency of this solvent composition.

In summary, methanol, particularly at 100% and 70% concentrations, generally shows more instances of ion enhancement, making it more effective in minimizing suppression effects compared to acetonitrile. Conversely, acetonitrile at 100% and 70% concentrations predominantly exhibits ion suppression, whereas 50% acetonitrile shows a mix but leans more towards ion enhancement. These findings suggest that methanol is generally more effective at mitigating matrix effects, thereby providing more reliable quantification of diuretics in whey protein.

Conclusion

In conclusion, this study explored the efficiency of different solvent extraction methods for detecting diuretics in whey protein using LLE-LC-MS/MS. Methanol and acetonitrile at varying concentrations (100%, 70%, and 50%) were evaluated for their ability to recover diuretic compounds and mitigate matrix effects. Methanol, particularly at 100% and 70% concentrations, consistently showed superior performance in recovering diuretics from whey protein. 100% methanol exhibited the highest recovery rates with moderate signal enhancement and minimal suppression, indicating its robust capability to reduce matrix interferences. Similarly, 70% methanol maintained balanced matrix effects and reliable recoveries, making it a dependable choice for diuretic analysis. These findings are consistent with several previous studies that highlighted methanol as an effective extraction solvent in drug analysis for dietary supplements using the LLE method. These studies reported high recovery rates and minimal matrix interferences when using methanol, corroborating our results.

In contrast, acetonitrile demonstrated more variability in matrix effects and generally lower recovery rates. For instance, 100% acetonitrile showed significant signal suppression, highlighting its limitations in effectively extracting diuretics from whey protein. Although 70% acetonitrile provided a more stable profile than other concentrations, it still exhibited variability of matrix effects, that affects its reliability compared to methanol. The study underscores the importance of solvent selection in minimizing matrix effects and ensuring accurate diuretic quantification in complex matrices like whey protein. Methanol’s stronger solvating power and polarity make it more effective in mitigating matrix interferences compared to acetonitrile. Future research should focus on optimizing solvent compositions and refining sample cleanup procedures to enhance the reliability and robustness of diuretic detection methods in complex food matrices. This approach will contribute to improved analytical accuracy and consistency in detecting diuretics in dietary supplements and other food products.

Table 2. The recoveries percentage of diuretic compounds for different types and ratio of solvents

Code	**Methanol**						**Acetonitrile**					
	100M		**70M**		**50M**		**100A**		**70A**		**50A**	
	Recovery (%)	**S.D (n=3)**	**Recovery (%)**	**S.D (n=3)**	**Recovery (%)**	**S.D (n=3)**	**Recovery (%)**	**S.D (n=3)**	**Recovery (%)**	**S.D (n=3)**	**Recovery (%)**	**S.D (n=3)**
D1	74.656	2.413	91.907	8.030	93.439	7.840	0.315	0.120	93.962	11.924	70.756	1.582
D2	85.981	1.576	94.492	6.793	83.281	5.099	1.228	0.076	92.525	0.076	62.809	0.024
D3	49.639	3.481	67.175	3.060	52.930	3.159	1.931	0.269	46.939	0.267	42.842	1.227
D4	90.905	1.505	77.391	4.843	46.766	2.422	0.135	0.071	86.346	0.071	56.714	0.094
D5	85.540	2.259	83.277	4.028	69.452	5.145	0.114	0.073	88.564	0.073	59.161	0.339
D6	96.211	1.968	93.633	1.944	67.166	5.074	3.234	1.145	90.391	1.145	59.821	1.254
D7	61.560	2.695	83.011	3.905	75.776	6.494	2.243	0.115	93.889	11.902	63.752	0.024
D8	81.815	1.555	88.994	3.791	74.038	8.843	0.205	0.139	81.059	17.557	57.564	1.223
D9	80.018	8.477	79.727	5.433	49.856	2.967	0.117	0.063	86.953	14.784	54.176	1.476
D10	84.539	2.801	82.715	3.508	66.956	5.777	9.267	6.176	83.706	14.859	58.458	0.604
D11	69.772	14.224	46.976	0.400	43.525	4.106	6.106	0.915	48.547	3.028	31.331	1.811
D12	61.691	7.293	82.007	6.015	69.386	5.616	2.872	1.637	76.813	14.256	55.944	4.218
D13	70.141	1.911	84.089	3.858	76.235	4.688	0.105	0.075	87.851	16.392	58.682	2.134
D14	80.489	2.480	87.083	6.582	76.487	7.332	0.157	0.048	87.049	18.280	57.448	2.119
D15	97.999	3.782	83.091	4.852	69.629	8.199	0.070	0.069	84.266	0.069	56.298	1.828
D16	89.469	3.960	93.767	5.782	84.230	6.016	0.131	0.096	91.555	0.096	68.689	2.086
D17	74.904	2.896	91.726	4.242	71.417	5.637	1.389	0.749	92.731	19.440	31.616	3.739
D18	95.495	4.291	88.546	4.724	69.593	3.161	3.147	0.101	92.717	0.101	61.496	0.971
D19	79.378	0.782	82.973	4.304	72.096	4.709	0.119	0.098	85.783	0.098	56.771	0.759
D20	99.735	5.800	91.913	5.305	66.915	5.378	7.869	1.635	93.319	1.635	61.185	0.828
D21	87.268	1.239	90.530	4.965	73.926	4.249	0.104	0.098	88.035	0.098	58.135	1.236
D22	54.226	0.704	61.987	2.549	46.865	2.159	1.534	0.364	69.749	0.364	52.107	1.610
D23	85.538	6.006	72.969	13.416	54.090	4.091	6.357	2.119	88.465	20.761	50.919	1.236

**S.D: standard deviation*

Table 3. The matrix effect percentage of diuretic compounds for different types and ratio of solvents

Code	**Methanol**						**Acetonitrile**					
	100M		**70M**		**50M**		**100A**		**70A**		**50A**	
	ME (%)	**S.D (n=3)**	**ME (%)**	**S.D (n=3)**	**ME (%)**	**S.D (n=3)**	**ME (%)**	**S.D (n=3)**	**ME (%)**	**S.D (n=3)**	**ME (%)**	**S.D (n=3)**
D1	12.115	3.523	7.834	2.061	47.156	2.477	27.438	28.641	-44.539	43.869	-3.956	6.231
D2	13.106	4.520	8.813	6.070	9.799	2.321	-46.424	8.812	-0.963	5.716	5.991	6.511
D3	-9.368	2.577	-3.395	8.447	0.772	3.739	-0.485	7.121	29.493	3.006	55.270	3.763
D4	19.087	3.354	11.220	11.124	18.623	3.701	19.637	69.442	-5.635	0.973	10.600	4.187
D5	16.116	3.986	10.050	8.577	0.887	2.179	-15.214	3.443	-6.043	2.054	5.773	3.662
D6	12.912	4.614	8.763	5.868	2.752	1.304	-31.514	7.897	-10.103	2.139	1.7995	4.429
D7	7.954	3.688	-70.185	11.026	-179.317	3.700	-32.723	6.137	-24.773	7.275	-171.026	23.498
D8	-38.626	7.446	10.548	6.701	49.889	4.556	-20.497	6.635	-1.016	1.635	68.654	1.171
D9	21.704	2.015	4.239	4.528	17.653	2.618	-27.671	5.081	-1.870	3.695	11.193	4.957
D10	14.077	3.400	-3.433	6.068	-0.320	3.790	-44.314	8.070	-8.293	0.070	11.116	5.648
D11	22.506	5.250	6.972	7.224	4.152	4.306	-36.565	5.339	-16.304	4.594	-0.164	1.111
D12	20.611	5.159	9.118	3.715	4.191	3.606	-21.342	4.188	3.779	1.831	11.297	5.729
D13	4.381	6.022	-11.374	5.120	-7.947	3.841	-47.013	7.899	-21.341	3.657	-8.241	8.060
D14	13.942	3.010	0.977	5.842	61.787	1.889	-35.593	9.473	-10.706	1.496	66.798	1.247
D15	8.762	0.572	4.667	5.792	0.709	3.393	-34.314	6.226	-6.619	3.323	1.192	4.401
D16	15.893	4.239	10.066	8.240	2.433	2.341	-66.284	11.609	-2.278	5.066	9.199	7.607
D17	10.397	4.543	7.700	4.368	-8.839	4.1814	-89.837	8.911	-10.347	3.189	0.726	7.678
D18	13.267	3.972	8.619	6.572	0.402	2.959	-23.992	2.938	-7.163	3.239	1.159	6.448
D19	13.385	3.673	8.529	6.867	0.639	3.450	-13.483	4.225	-12.119	3.854	4.083	6.048
D20	12.378	2.825	7.602	6.754	0.217	3.922	-30.188	6.810	-8.583	1.977	2.518	6.044
D21	16.810	3.321	10.066	9.538	7.772	5.181	-10.668	3.612	4.155	1.888	17.572	4.540
D22	20.028	5.472	12.750	10.292	0.888	2.435	-15.214	9.593	-28.567	10.443	4.276	10.45
D23	-6.231	8.529	-2.911	6.049	-9.031	16.440	-24.192	11.510	-11.720	9.076	-10.523	4.132

**ME: matrix effect*

**S.D: standard deviation*

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