Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Based on the 4D Label-free protein qualitative analysis method and coomassie brilliant blue method quantitative determination of protein content to improve the quality standards of Xiangdan injection

Min Xu
Chengdu University of Traditional Chinese Medicine
Zhangkai Ye
Xinjiang Normal University
Yuchen Chen
Chengdu University of Traditional Chinese Medicine
Jia Cai
Chengdu University of Traditional Chinese Medicine
Shunpeng Zhu
Chengdu University of Traditional Chinese Medicine
Qiang Yang
Sichuan Shenghe Pharmaceutical Co. Ltd.
GuoLiang Wen
Sichuan Shenghe Pharmaceutical Co. Ltd.
Ruizhen Huang
Hospital of Chengdu University of Traditional Chinese Medicine
Liang Zhang
Chengdu Institute for drug control, Chengdu 610045, Sichuan, China
Qiang Ye
yeqiang@cdutcm.edu.cn

Chengdu University of Traditional Chinese Medicine

Article

Keywords: xiangdan injection, trace protein, 4D label-free proteomics, qualitative experiments, quantitative experiments, improvement of quality standards

Posted Date: June 11th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4483658/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Xiangdan injection (XDI), as a common Chinese medicine injection (CMIs), is mainly used clinically to activate blood circulation, relieve blood congestion and dilate coronary arteries. However, allergic reactions occasionally occurred during the use of XDI, which could be related to the protein in XDI. Therefore, we conducted research on the commercial XDI and tested it according to the requirements of the Chinese Pharmacopoeia for CMIs. Firstly, 4D-Label-free quantitative proteomics technique was used to identify the samples of XDI and Salvia miltiorrhiza (SM), eleven identical proteins were found. The results indicated that XDI contained trace proteins which come from SM with a molecular weight range of 15-181KD. Then the qualitative experiments about proteins were carried out via the sulfosalicylic acid (SA) method and the tannic acid (TA) method, which were required in the Chinese pharmacopoeia. To our surprise, the qualitative experiments in the Chinese pharmacopoeia could not precise identify the proteins in XDI. Unexpectedly, the reason is that the excipient Tween 80 in XDI would cause a false-positive reaction. Therefore the proteins in XDI were inspected qualitatively and quantitatively after eliminating false-positive reactions. The protein content in XDI was determined by coomassie brilliant blue method. It was discovered that the protein concentration was 3.038 mg/ml, which would not generate a positive reaction. Finally the combination of qualitative and quantitative protein experiments provide an exclusive identification method for XDI, which could promote the quanlity standards of XDI and guarantee the clinic safety.

Introduction

Chinese medicine injections (CMIs) refer to the injection dosage form made according to the guidance of the traditional Chinese medicine (TCM) theory, adapting modern science and technology to extract the active ingredients or compounds from Chinese medicine. Comparing to the traditional Chinese medicine dosage forms CMIs improved the bioavailabilities and therapeutic effects to treat acute and severe diseases, changing the traditional acquaintaince that Chinese medicine was only suitable for "slow" diseases [1]. Especially in the treatment of cardiovascular diseases, respiratory diseases and tumors, CMIs have a wide range of clinical applications [2-13]. Although CMIs were widely used in clinical practice, the occasionally adverse reactions severely limited the development of CMIs. A statistical data about 32309 people used CMIs in one hospital per year there were 91 cases of adverse reactions, the adverse reaction rate was only 0.28 percent [14,15]. Though the incidence of adverse reactions was rather low overall, they significantly affected the reputation and the use of CMIs. Allergy, gastrointestinal reactions and fever are the most common clinical adverse reactions to CMIs. Few adverse reactions such as haemolysis, haematopenia, anaphylaxis, dyspnoea and anaphylactoid reactions may lead to potentially fatal [16]. The complex composition of CMIs and the simultaneous use of polypharmacy could lead to the occurrence of adverse reactions [17,18]. The vast majority of CMIs are compound preparations with complex ingredients. Some impurities, such as pigments, tannins, starch and proteins, are present in the medicinal solution as colloidal particles [19]. Proteins, a class of macromolecules made up of amino acids, are a general allergen. The human immune system usually produces an immune response to

specific structures or sequences in proteins. Therefore, excessive protein content in CMIs can easily cause allergic reactions.

The Chinese Pharmacopoeia (2000 Year Edition) firstly proposed the examination of relevant substances in CMIs, which included proteins, tannins, resins, oxalates, and potassium ions. But there were some false positives [20]. In Appendix of Chinese Pharmacopoeia (2010 Year Edition) has clearly stipulated that all CMIs should be examined for proteins [21]. The aim is to improve the safety of CMIs and to reduce the risks associated with medication. Therefore, it is necessary to find an exclusive examination method for CMIs that excludes the interference of false positive reactions.

XDI is a traditional CMI approved in the National Ministerial Standard (WS3-B-3289-98), which is made from *Salvia miltiorrhiza* (SM) and *Dalbergia odorifera* (DO). XDI with a history of over twenty years in traditional Chinese medicine has been widely used in clinical practice mainly for pain relief and promotion blood circulation [22,23]. SM contains chemical components such as tanshinones, salvianolic acids, volatile oils, polysaccharides and nitrogen-containing compounds, which have anticoagulant, anti-inflammatory, antioxidant and organ-protective effects [24-31]. The main chemical constituents of DO are volatile oils and flavonoids, which have pharmacological activities such as vasodilation, increased coronary artery flow, blood lipid regulation and blood pressure reduction [32-34]. The combination of the two drugs enhances the efficacy of the drug, but the incidence of adverse reactions occasionally occured with more and more usage of XDI. According to statistics, from January 2006 to December 2018, there were totally 400 cases of adverse reactions about 33 cases per year. These patients developed allergic reactions, so the allergens in XDI need to be strictly controlled in allergens [35,36]. Our research used 4D-Label-free quantitative proteomics technology to identify the proteins in SM and XDIs. The results showed that both SM and XDIs contained some trace proteins and that the vast majority of the proteins in XDIs were from SM. We continued with the qualitative and quantitative study of the proteins in XDI to analyzed the residual proteins associated with each critical step in the whole preparation process. Through strictly controlling the protein content of XDI, the quality standard of XDI was improved and the occurrence of allergic reactions could be reduced significantly. In this article, the protein in XDI was detected by qualitatively and quantitatively to improve the quality standard of XDI, which would assure the safety of clinical use of the drug.

Materials And Methods

Chemicals and materials

Coomassie brilliant blue G250 (CBBG250), Ethanol, Phosphoric acid (PA), Sulfosalicylic acid, and Tannic acid were purchased from Chengdu Cologne Chemical Corporation. Bovine serum protein (BSA) was purchased from Beijing Solabao Technology Corporation. All other chemicals used in this study were of analytical grade.

Sichuan Shenghe Pharmaceutical Co. provided the samples of XDI as well as the Chinese medicines SM and DO used in the production process.

4D-Label-free Quantitative Proteomics

In order to determine what proteins are in the injection, the group used 4D-label-free quantitative proteomics technology to carry out the study. The analysis process of this project mainly included two stages: a mass spectrometry experiment and data analysis. The analysis process of the mass spectrometry experiment mainly included protein extraction, peptide digestion, chromatographic classification, liquid chromatography-tandem mass spectrometry (LC-MS/MS) data collection, database search, and other steps, according to the database for bioinformatics analysis. The analysis mainly included identification of the number of analyses, expression difference analysis, functional analysis, and so on (Figure 1).

Figure 1. Flowchart of 4D-Label-free quantitative proteomics experiments.

Qualitative protein examination methods

According to the requirements of the Chinese Pharmacopoeia (2020 Year Edition), take 1.0 mL of injection and add 1.0 mL of freshly prepared 30% SA solution and mix evenly. After being left for 5 minutes, the sample should not show any precipitation reaction. If the injection contains ingredients that can precipitate in the case of acid, we could add one to three drops of TA solution. The sample also should not show any precipitation reaction [37].

Because of the presence of Tween 80 in the excipients of XDI, the use of TA will generate precipitation and cause a false positive reaction. So the addition of Tween 80 could only be used in SA method for protein detection. However, samples without Tween 80 in this experiment can be inspected by the TA method, which increases the credibility of this experiment [37].

Protein examination of medicinal liquid

The 1.0 ml of SM medicinal intermediates (S1, S2, S3, S4, S5, S6, S7, S8) and DO medicinal intermediate in the process were precisely measured in tubes and added in of 1.0 ml of 30% SA or 5%TA. The next step was the observation of the precipitation of the solution [37].

Protein examination of Tween 80

The 1.0 ml of a mass fraction of 0.5% Tween 80 which from six different manufacturers were accurately measured in tubes and added to 1.0 ml of 30% SA or 5% TA. The next step was the observation of the precipitation of the solution [37].

Meanwhile, since TA plus Tween 80 would produce precipitation, this group also explored the minimum concentration of Tween 80 that did not generate precipitation with TA. TA at 0.5% was used as the initial

concentration for gradient dilution to find the minimum concentration that did not generate precipitation with TA.

Quantitative protein determination methods

Methods of quantitative determination of proteins

According to the Chinese Pharmacopoeia, there were six methods for protein quantitative examination. This group pre-screened these six methods and finally determined that the protein content was determined by the Coomassie brilliant blue method, and a methodological examination was done.

Methodological examination of the Coomassie brilliant blue method

Preparation of standard solution

The 10.0 mg of BSA was precisely weighed in a 10.0 mL volumetric bottle and dissolved in of ultrapure water. Then we could obtain the 1.0 mg/ml BSA standard solution.

Preparation of test product solution

The 1.0 ml of Sheng He's XDI and SM process intermediates (S1-S8) were precisely measured in a volumetric bottle and diluted in of ultrapure water. Then we could obtain the test product solution.

Investigation of linear relationship and linear range

The determination was made by the CBBM. 0.0 ml, 0.01 ml, 0.02 ml, 0.04 ml, 0.06 ml, 0.08 ml and 0.1 ml of the standard solution were accurately measured and placed in a stoppered test tube. Respectively, 0.1 ml of ultrapure water and 5.0 ml of the acidic staining solution were added. The absorbance of BSA was measured using an ultraviolet/visible spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) at 595 nm. And the tube S0 was used as a blank at the same time. The linear regression equation was calculated by taking the concentration of BSA solution (mg/ml) as the horizontal coordinate and the absorbance corresponding to it as the vertical coordinate.

Taking the concentration of BSA (mg/ml) as the horizontal coordinate and its corresponding absorbance as the vertical coordinate, a linear regression equation $y = 0.0431x + 0.0202$, $r = 0.9996$ was obtained (Figure 2). The experimental results showed that the range of bovine serum albumin in the concentration of 3.921 μg/ml–19.607 μg/ml in the linear relationship was well.

Figure 2. Protein standard curve.

Precision test

A defined volume of BSA standard solution was accurately withdrawn and measured as described in "Investigation of linear relationship and linear range". The measurement was repeated six times, the

absorbance (A) was measured and the RSD value was calculated. The results showed that RSD = 0.00% (Table 1), indicating that the precision of the system was good.

Table 1. Results of the Coomassie Brilliant Blue method precision test.

Solution stability test

The test solution in "Preparation of test product liquid" was measured at room temperature for 0, 5 and 10 minutes. According to the method under “Investigation of linear relationship and linear range”, measured absorbance (A) and calculated the RSD value. The results showed that the RSD was 0.059% (Table 2), indicating that the stability of the sample was good after mixing with a Coomassie Brilliant Blue solution at room temperature for 10 minutes.

Table 2. Coomassie Brilliant Blue method stability test results.

Sample recovery test

Six XDI samples of 0.5 mL each were taken and 0.5 mL of BSA standard solution at 80%, 100% and 120% of the sample solution concentration was accurately added. Each sample was paralleled three times, and the absorbance (A) was measured according to the method in “Investigation of linear relationship and linear range”. Then the recovery rate of addition was calculated. The results showed recoveries ranging from 101.14% to 105.298% (Table 3), indicating the good accuracy of the method.

Table 3. Results of Coomassie Brilliant Blue spiked sample recovery test.

Repeatability test

Six samples of the same batch of XDI were precisely measured, the test solution was prepared according to “Preparation of test product liquid”. And the absorbance (A) was measured according to the method under “Investigation of linear relationship and linear range”. Then the RSD value was calculated. The results showed an RSD of 4.53% (Table 4), indicating good repeatability of the system.

Table 4. Coomassie brilliant blue repeatability test results.

Sample inspection

The intermediate liquids of XDI and SM were precisely measured, the test solution was prepared according to “Preparation of test product liquid”, and the absorbance (A) was measured according to the method under “Investigation of linear relationship and linear range”. Then the concentration was calculated.

Results

4D-Label-free Proteomics Methods

Identification and quantification results of the ProteinGroups A and B

This experiment divided into two groups: Group A was SM medicinal herb, and Group B was XDI (Table 5). Firstly, proteins were extracted from SM medicinal herb and were identified using LC-MS. A total of 59105 spectra were obtained from SM medicinal herb. After comparison with the protein database of SM medicinal herb on internet, 1743 spectra were successfully matched; the number of 682 peptide segments were obtained from SM medicinal herb and 646 peptide segments were successfully matched; 173 proteins were qualitatively identified in SM medicinal herb while 171 proteins were quantitatively identified (Table 6 and Figure 3). There were 4, 9, and 1 ProteinGroups of the three XDIs samples in Group B1, B2 and B3 respectively**,** therefore the identified ProteinGroup in group B were 14 (Table 7).

Table 5. Sample grouping.

Table 6. Identification and quantification results of the ProteinGroup A.

Figure 3. Identification and quantification results of the ProteinGroup A.

Table 7. Identification and quantification results of the ProteinGroup A and B.

The same proteins in ProteinGroup A and B

In order to analyze the same proteins between two groups, the overlap of proteins were identified by Wayne diagram analysis (Figure 4). There were eleven same proteins in two groups. Respectively they were Cytochrome P450 CYP98A78, Cytochrome P450 CYP82D70, Enoyl-ACP Reductase, ABC Acyl Transporter, Carboxyltransferase beta subunit of Heteromeric ACCase, Universal stress protein 1, Universal stress protein 26, ATP synthase subunit beta, chloroplastic, ABC-type xenobiotic transporter, 14-3-3-like protein C (Fragment), and Late embryogenesis-like protein. The conformations of the eleven proteins were described in the PBD database (Table 8 and Figure 5).

Figure 4. Venn diagram of proteins identified between all sample groups.

Table 8. The molecular weight of the eleven same proteins

Figure 5. Conformations of the eleven same proteins (Database: PDB: https://www.rcsb.org/).

171 proteins were successfully identified in SM and 13 proteins were identified in XDI. There were eleven identical proteins in both XDI and SM, which indicated that almost all the proteins in XDI were from SM. This trial not only identified the presence of proteins in XDI, but also measured the molecular weight in the range of 15-181 KD. The 4D-Label-free Proteomics Method is an effective method which could rapidly detect the proteins in XDI and the results provides a reference for later qualitative experiments.

Results of qualitative protein examination

The examination standards for injections in the Chinese Pharmacopoeia

According to the Chinese Pharmacopoeia (2020 Year Edition), there are two qualitative protein examination methods for injections. One is sulfosalicylic acid (SA) method, the other is tannic acid (TA) method [37]. Both require that the samples do not precipitate with the reagents.

Classification of qualitative examination on protein in XDI

The XDI process includes the preparation of SM and DO. Figure 6 shows the whole process and some key sampling points.

Figure 6. The whole process flow diagram of XDI and crucial steps of sampling points.

Through analyzing the whole process flow diagram of XDI, there were three situations could cause positive reactions of protein. The first situation was the medicinal liquid, including the medicinal liquid of SM and DO; the second situation was the excipient Tween 80; the third situation was the medicinal liquid plus Tween 80. In order to find the reason of the positive reaction, our group carried out inspections based on these three situations (Figure 7).

Figure 7. Three situations

Qualitative examination results for three situations

The first situation——protein examination of medicinal liquid

DO medicinal liquid

Our team has successfully produced qualified XDI according to the standards of the Chinese Ministry of Health. From the preparation process of DO, we selected one sample. The sample (D1) was detected by SA and TA methods. The results indicated that D1 did not precipitate with SA and TA. (Figure 8).

Figure 8. DO medicinal liquid did not precipitate with SA and TA. (a) SA+DO medicinal liquid; (b) TA+DO medicinal liquid

SM medicinal liquid

From the preparation process of SM, we selected eight samples of crucial steps which probably contained the protein of XDI. These samples (S1, S2, S3, S4, S5, S6, S7, S8) were tested by SA and TA methods. And the results showed that these samples of intermediates of SM did not generate precipitation with SA and TA (Table 9,10).

Table 9. The samples of intermediates of DO medicinal liquid and SM medicinal liquid

Table 10. Qualitative results of SM medicinal liquid

Briefly, neither the DO nor the SM medicinal liquid generated any positive reaction. This results indicated that the samples of intermediates of medicinal liquid did not generate the positive reactions.

The second situation——Tween 80

The interference of Tween 80 in protein qualitative examination

There were six kinds of Tween 80 from different manufacturers (Table 11). We utilized SA and TA methods for examining as well. The observation was that they were not generate precipitation with SA. But they all generate precipitation with TA. This experiment indicated that Tween 80 did not generate a positive reaction with SA (Table 12).

Table 11. Sample number of the six Tween 80.

Table 12. The qualitative results of six kinds of Tween 80 from different manufacturers.

This experiment conducted qualitative experiments on Tween 80 which produced by different manufacturers. Tween 80 was shown not to generate a positive reaction with SA. Due to the structure and properties of Tween 80, it would reacted with TA leading to interference of qualitative examination on protein.

Investigation of the critical concentration of Tween 80

Because TA and Tween 80 could generate precipitation, this research explored the critical concentration which Tween 80 plus TA did not generate precipitation. The 0.5% Tween 80 as the initial concentration was diluted to 0.05%, 0.025%, 0.0125%, 0.00625%, 0.005%. Finally, concentrations of 0.00625% and below were found not to generate precipitation. The critical concentration of precipitation was 0.00625% (Table 13).

Table 13. Results of different concentrations of Tween 80 with TA

The third situation—Protein examination of medicinal liquid + Tween 80

DO medicinal liquid+ Tween 80

The team added Tween 80 to each intermediate separately according to the requirements of the process production. The sample (D1+Tween 80) was tested by SA methods. The result showed that the sample (D1+Tween 80) did not precipitate with SA. The sample (D1+Tween 80) was tested by TA methods. The result showed that the sample (D1+Tween 80) would precipitate with TA (Figure 9).

Figure 9. Qualitative examination results of DO medicinal liquid + Tween 80. (a) SA+DO medicinal liquid; (b) TA+DO medicinal liquid

SM medicinal liquid + Tween 80

The samples (S1+Tween 80, S2+Tween 80, S3+Tween 80, S4+Tween 80, S5+Tween 80, S6+Tween 80, S7+Tween 80, S8+Tween 80) was tested by SA methods. There was no precipitation of the samples (S1+Tween 80, S7+Tween 80, S8+Tween 80) with SA. But the samples (S2+Tween 80, S3+Tween 80, S4+Tween 80, S5+Tween 80, S6+Tween 80) generated precipitation with SA (Table 14 and Figure 10).

Table 14. Qualitative examination results of *SM* samples.

Figure 10. Qualitative examination results of the samples with SA.

The samples (S1-1, S2-1, S3-1, S4-1, S5-1, S6-1, S7-1, S8-1) was tested by TA methods. The result showed that the samples all generated precipitation with TA.

Table 15. Qualitative examination results of the three situations.

The three situations were detected by SA and TA method in turn (Table 15). The results showed that SM medicinal liquid did not generate positive reaction with SA. Tween 80 did not generate positive reaction with SA. However, the mixture of SM medicinal liquid and Tween 80 would generate a positive reaction with SA. We speculated that some components of SM may have reacted with Tween 80, which causing a false positive reaction. Furthermore, no precipitation was observed with TA at Tween 80 concentrations of 0.00625% or less.

Results of quantitative protein determination

Results of Samples quantitative protein determination

The Coomassie brilliant blue method (CBBM) is a common method for determining protein content. This method establishes a standard curve based on the absorbance and concentration of the protein reference solution. And the protein content of the samples was calculated from the standard curve. However, the team analyzed the XDI process and found that XDI simply used a distilled water liquid of DO. As we all know, proteins are not distilled by water vapor. Therefore, the group focused on the protein content of SM medicinal liquid. The group measured the absorbance of the samples and calculated the protein content of the sample (Table 16). The samples for this experiment came from the process intermediates of XDI (Figure 6).

Table 16. Results of protein concentration determination.

Figure 11. Variation of protein content in the production process of XDI

After several treatments, the protein in the SM medicinal liquid was found to be significantly reduced (Table 16 and Figure 11). Protein mass decreased from 12582.391 mg to 2406.718 mg. The concentration operation and the first alcohol precipitation operation had the greatest influence on the protein content. However, the protein content in samples S3 to S8 fluctuated within a certain range. We speculated that this phenomenon might be caused by the aggregation of proteins in the solution. We still need to explore the specific reasons for this.

Table 17. Determination of the concentration of proteins in XDI.

The final concentration of protein in the finished product of XDI was determined to be 3.038 mg/ml by repeated measurements (Table 17). Based on the qualitative and quantitative experimental results, it can be seen that excessive protein concentration in XDI could lead to precipitation. The group analyzed the protein concentrations of eight process intermediates. And we found that it would not generate precipitation when the protein concentration was below 6.5 mg/ml (Table 16,17 and Figure 11).

Discussion

Qualitative results based on 4D Label free quantitative protein technology

Our research first applied 4D label-free quantitative protein technology to XDI protein detection. 4D-Label-free quantitative protein technology can rapidly identify the proteins contained in XDI. Relying on the 4D-Label-free quantitative protein technique, it was confirmed that the proteins in XDI were mainly derived from SM. In addition, we also measured the molecular weights of these proteins in the range of 15-181 KD, with an average molecular weight of 50 KD. We used the 4D-Label-free quantitative protein technique to identify the proteins in XDI qualitatively. However, it was not used to quantify the proteins in the injection. Therefore, the next step will be to investigate this method in more detail and use this technique to quantitatively determine the proteins in XDI.

The results of qualitative examination

During our research, we discovered that XDI contains a tiny amount of protein. Therefore, we conducted several qualitative examination of XDI according to the Chinese Pharmacopoeia (2020 Year Edition). The results showed a positive reaction between the marketing XDI and SA. Some review of the literatures has shown that different concentrations of Tween 80 will affect the SA method. When the sample contains Tween 80, it will cause false positives [38]. However, the excipient in XDI is Tween 80 and the positive interference need to be excluded from this assay method.

The possible situations of positive reactions of XDI were divided into three situations. We carried out qualitative experiments on each of them. The first situation was the medicinal liquid, which was discovered not to generate a positive reaction with SA and TA. In the second situation, which was Tween 80, SA did not react positively with Tween 80, but TA reacted positively with Tween 80. The third situation was the medicinal liquid plus Tween 80. The results indicated that the samples (S1+T80, S7+T80, S8+T80) did not generate precipitates, while the other samples (S2+T80, S3+T80, S4+T80, S5+T80, S6+T80) generated precipitates.

Analyzing the results of the qualitative examination as a whole, we found that neither the medicinal liquid nor Tween 80 would react positively with SA. Whereas the addition of Tween 80 to the medicinal liquid generated a positive reaction. From the results, we were able to deduce that Tween 80 was the cause of the false positive reaction. Interestingly, however, we detected that when we doubled the amount of SA,

there was no precipitation reaction. We speculated that perhaps the addition of an extra 1.0 ml of SA would dilute the medicinal liquid, so we then tested the protein content. For this experimental result, we speculated that certain components in the SM medicinal liquid might react with Tween 80 and cause a positive reaction. Of course, more research was needed to understand the specific reasons for this. Furthermore, if Tween 80 that is added to XDI at a concentration below 0.00625%, it may be not precipitate with TA.

The results of quantitative determination

We screened six quantitative methods for protein detection in the Chinese Pharmacopoeia (2020 Year Edition). Finally, the decision was made to determine the protein in XDI by using the CBBM. It should be noted that the addition of coomassie brilliant blue sulotion to the sample was unstable within one hour. But it relatively stable within the initial ten minutes. Therefore, we needed to complete the measurement in ten minutes. After repeated measurements, the protein content in XDI was 3.038 mg/ml. Moreover, our team produced XDI according to the process flow of the Ministry of Health of the People's Republic of China. The XDI process flow included the preparation process of SM and DO. From the preparation process of SM, we selected eight samples of crucial steps which probably contained the protein of XDI. From the preparation process of DO, we selected one sample. We also determined the protein content of these process intermediates by CBBM. Monitoring of protein changes in the production process by measurement of the protein content of these intermediates.

From Figure 11 and Table 16, it can be clearly seen that most of the proteins have been removed by multiple alcohol and water precipitation in the process of XDI. The protein content in XDI was gradually reduced after several steps. And the total content was reduced from the initial 12,582.391 mg to 2,406.718 mg. The effect of the first alcohol precipitation on protein removal was particularly pronounced. Besides, the protein concentration obtained in the final product of XDI was only 3.038 mg/ml. This concentration of protein belongs to trace proteins, which not enough to cause a positive reaction. Analyzing the qualitative and quantitative results together, we observed that the lowest concentration of this samples (S2+T80, S3+T80, S4+T80, S5+T80, S6+T80) which produced a positive reaction was 6.884 mg/ml. So we concluded that if the protein concentration did not exceed 6.50 mg/ml, it would not generate a positive reaction.

In summary, by analyzing all the results, we discovered that the SA method in the Chinese Pharmacopoeia (2020 Year Edition) was susceptible to false positive interference. Therefore, we hope that the research will provide a reference for the identification of XDI and improve its quality standard. We suggest that the examination of XDI should not only include the qualitative examination, but also the quantitative examination. Meanwhile, the protein content of a qualified product should not be more than 6.50 mg/ml. The addition of 2.0 ml of SA was also observed to eliminate the precipitate, but the specific reason for this is still under investigation. Another problem was that SM liquid itself did not react with SA, but the precipitation occurs after plus Tween 80. We speculated that some components in SM liquid

may react with Tween 80 to form new substances. Then it will react with SA to cause precipitation. But the specific reasons need further investigation.

Conclusion

We used 4D label-free quantitative protein technology to identify the protein in XDI and carried out qualitative experiments on XDI which using the Chinese Pharmacopoeia method. However, we discovered that the examination of CMIs using the Chinese Pharmacopoeia method was not very accurate. It was easy to lead to false-positive reaction. Subsequently experiments revealed that Tween 80 was the reason of the false positive reaction of XDI. Furthermore, through quantitative experiments, we determined the protein content in XDI was 3.038 mg/ml. By analyzing the proteins in XDI qualitatively and quantitatively, we can provide an exclusive identification method for XDI. The detection of XDI should not only include qualitative examination, but also quantitative examination. At the same time, quantitative examination requires that the protein concentration in XDI does not exceed 6.50 mg/ml. This article improves the quality standard of XDI through quantitative protein analysis. This will provide a reliable reference for the safety of clinical usage in XDI. Our research used XDI as an example to improve its quality standards. We hope that other CMIs can refer to this model to improve quality standards. The research provides an idea for research to standardize the use of CMIs.

Abbreviations

XDI Xiangdan injection

CMIs Chinese Medicine injections

CBBG250 Coomassie brilliant blue G250

SM Salvia miltiorrhiza

DO Dalbergia odorifera

SA Sulfosalicylic acid

TA Tannic acid

PA Phosphoric acid

BSA Bovine serum protein

Declarations

Data Availability: The data presented in this study are available upon request from the corresponding author

Acknowledgments

Thanks to all participants and researchers involved in this study.

Author Contributions

Y. Q.: Experimental design, methodology validation, manuscript revision and funding acquisition; L. Z. and R.H.: Resources, supervision, Access to funds; M. X. and Z. Y.: Design of the experimental procedure, Validation of methodology, data processing, writing of the manuscript; Y.C.: Data processing, picture drawing; J. C. and S. Z.: Methodological validation, market research. Q. Y and G. W.: Process technology support, validation of experimental results. All the authors have read and approved the final manuscript.

Funding

This research was funded by the National Natural Science Foundation of China (grant number 81891012), Chengdu University of Traditional Chinese Medicine to improve the quality standard of Xiangdan injection (grant number 301023011). The author would like to express their special thanks to Sichuan Shenghe Pharmaceutical Co., Ltd. for providing process technology support.

Conflicts of Interest

All authors declare no conflict of interest regarding the present work and they have no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

References

1. Wu, Q., Zhang, X. & Song, Y. The Current Situation and Outlet of Traditional Chinese Medicine Injection. *Chinese and Foreign Medical Research***17**, 4 (2019).
2. Wang, K. *et al.* Comparative Efficacy of Tonic Class Chinese Herbal Injections for Treating AcuteMyocardial Infarction: a Network Meta-analysis. *Chinese Journal of Pharmacoepidemiology***28**, 11 (2019).
3. Hu, X. & Wei, F. Research progress in the treatment of common cardiovascular diseases with traditional Chinese medicine injections. *Chinese Journal of Clinical Rational Drug Use***000**, 172-173 (2015).
4. Wei, B. Cost-effectiveness analysis of three traditional Chinese medicine injections in the treatment of acute cerebral infarction. *Chinese and Foreign Medical Research***13**, 2 (2015).
5. Wang, C. *et al.* Survey of active components in commonly-used Chinese materia medica injectionsand related Chinese materia medica for cardiovascular disease. *Chinese Traditional and Herbal Drugs***46** (2015).
6. Fan, Y. Thinking about Treating Disease of Respiratory System by TCM Injection. *Western Journal of Traditional Chinese Medicine***28**, 3 (2015).

7. Huang, Y. Discussion on the stability of compatibility between commonly used traditional Chinese medicine injections and various antibacterial drugs. *Chinese and Foreign Medical Research***000**, 149-150 (2013).
8. Qian, S. Clinical efficacy analysis of Huachansu injection in the treatment of advanced malignant tumors. *Electronic Journal of Clinical Medical Literature***4**, 19170-19171 (2017).
9. Cao, X. Clinical efficacy of compound Sophora flavescens injection in relieving pain in patients with malignant tumors. *Guide of China Medicine***16**, 2 (2018).
10. Jiang, R. & Zhu, F. The effect of Shenqi injection as an adjuvant chemotherapy on the quality of life of patients with advanced lung cancer. *Journal of Emergency in Traditional Chinese Medicine***23**, 2 (2014).
11. Li, Q. *A dissertation submitted to Huazhong University ofScience and Technology for the Degree of Doctor of Medicine*, Huazhong University of Science and Technology, (2017).
12. Tian, J., Jia, Z. & Chen, X. Clinical observation of the effect of Aidi injection on immune function in patients with advanced tumors. *Journal of Guiyang College of Traditional Chinese Medicine***34**, 80-81 (2012).
13. Yang, X. Clinical observation of the effect of kanglaite injection on quality of life and immune function in patients with advanced malignant tumours. *Shanxi Journal of Traditional Chinese Medicine***29**, 2 (2013).
14. Huang, R. *et al.* Safety of traditional Chinese medicine injection based on spontaneous reporting system from 2014 to 2019 in Hubei Province, China. *Scientific reports***11**, 8875, doi:10.1038/s41598-021-88339-9 (2021).
15. Zhang, M. Nursing intervention for adverse reactions of traditional Chinese medicine injections. *Hubei Journal of Traditional Chinese Medicine***37**, 2 (2015).
16. Zhang, L. *et al.* Adverse reaction reports and measures analysis of traditional Chinese medicine injections. *The Journal of Medical Theory and Practice* (2023).
17. Chen, X. *et al.* Study on False Positive Results with Protein Examination in Guanxinning Injection. *Modern Chinese Medicine***017**, 1212-1215 (2015).
18. Huang, L. The Study on safety re-evaluation of post-marketingShenwe iShenmai injection. (2020).
19. Wang, L. & Yuan, Y. Issues to consider in the clinical use of traditional Chinese medicine injections. *Chinese Remedies & Clinics***5**, 1 (2005).
20. Commission, C. P. *Chinese Pharmacopoeia 2000 edition*. (Chinese Pharmacopoeia Commission 2000).
21. Commission, C. P. *Chinese Pharmacopoeia 2010 edition*. (Chinese Pharmacopoeia Commission, 2010).
22. Yang, L. *et al.* Simultaneous determination of nine constituents in Xiangdan Injection by HPLC. *Chinese Traditional Patent Medicine***42**, 5 (2020).

23. Li, Y., Liu, Z., Zhao, Y., Chen, C. & Wang, B. Network Analysis of Main Active Ingredients-Target-Pathway of Xiangdan Injection inTreatment of Coronary Heart Disease. *Chinese Pharmaceutical Journal***53**, 9 (2018).
24. Dong, R. & Zheng, Y. The Isolation and Identification of Tanshinone Components in Tanshin. *Jilin Normal University JournalNatural Science Edition***25**, 100-101 (2004).
25. Li, Y., Yang, D., Su, B., Du, G. & Lv, Y. Simultaneous Detection of 14 Hydrophilic Components in Salvia miltiorrhiza by UPLC. *Herald of Medicine***38**, 6 (2019).
26. Yang, J. *et al.* Research progress on anti-inflammatory pharmacological action of components of Salvia miltiorrhiza and its preparations. *Drug Evaluation Research***44**, 11 (2021).
27. Zhong, G. *et al.* Chemical Characteristics of Salvia miltiorrhiza (Danshen) Collected from Different Locations in China. *Journal of Agricultural & Food Chemistry***57**, 6879-6887 (2009).
28. Li, C. *et al.* Synergistic Effect of Xiangdan Injection and Qingkailing Injection on Organ InjuryCaused by Acute Inflammation and Thrombosis in Rats. *Chinese Journal of Experimental Traditional Medical Formulae*, 027 (2021).
29. Feng, K. *et al.* Chemical components and pharmacological action for Salviae Miltiorrhizae Radix etRhizoma and predictive analysis on quality markers. *Chinese Traditional and Herbal Drugs***53**, 10 (2022).
30. MEIm, X. *et al.* Danshen: a phytochemical and pharmacological overview. *Chinese journal of natural medicines***17**, 59-80, doi:10.1016/s1875-5364(19)30010-x (2019).
31. Shi, B. *et al.* Pharmacokinetics of 13 active components in a rat model of middle cerebral artery occlusion after intravenous injection of Radix Salviae miltiorrhizae-Lignum dalbergiae odoriferae prescription. *Journal of separation science***43**, 531-546 (2020).
32. He.X *et al.* Research Progress on Chemical Constituents and Pharmacological Effects of Dalbergia odorifera. *Modern Chinese Medicine*, 024 (2022).
33. Li.Z. Studies on The Jiangxiang of Xiangdan injection. *Guangdong Pharmaceutical College* (2009).
34. Son, N. T. A Review on the Medicinal Plant Dalbergia odorifera Species: Phytochemistry and Biological Activity. *Evidence-Based Complementray and Alternative Medicine,2017,(2017-12-4)***2017**, 1-27 (2017).
35. Qin, Y. & Cheng, M. Analysis of 387 Cases of Adverse Reactions of Xiangdan Injection. *Chinese Journal of Clinical Rational Drug Use***10**, 2 (2017).
36. Liu, G. Analysis of 65 Cases of Adverse Reactions of Xiangdan Injection. *Jiangxi Medical Journal***54**, 2 (2019).
37. Commission, C. P. *Chinese Pharmacopoeia 2020 edition*. (Chinese Pharmacopoeia Commission, 2010).
38. Zu, J. *et al.* Influence of Polysorbate 80 on Examining Protein in Chinese Medicinal Injections withPharmacopoeia Method. *Drug Standards of China*, 3 (2015).

Tables

Tables 1-17 is available in the Supplementary Files section.

Figures

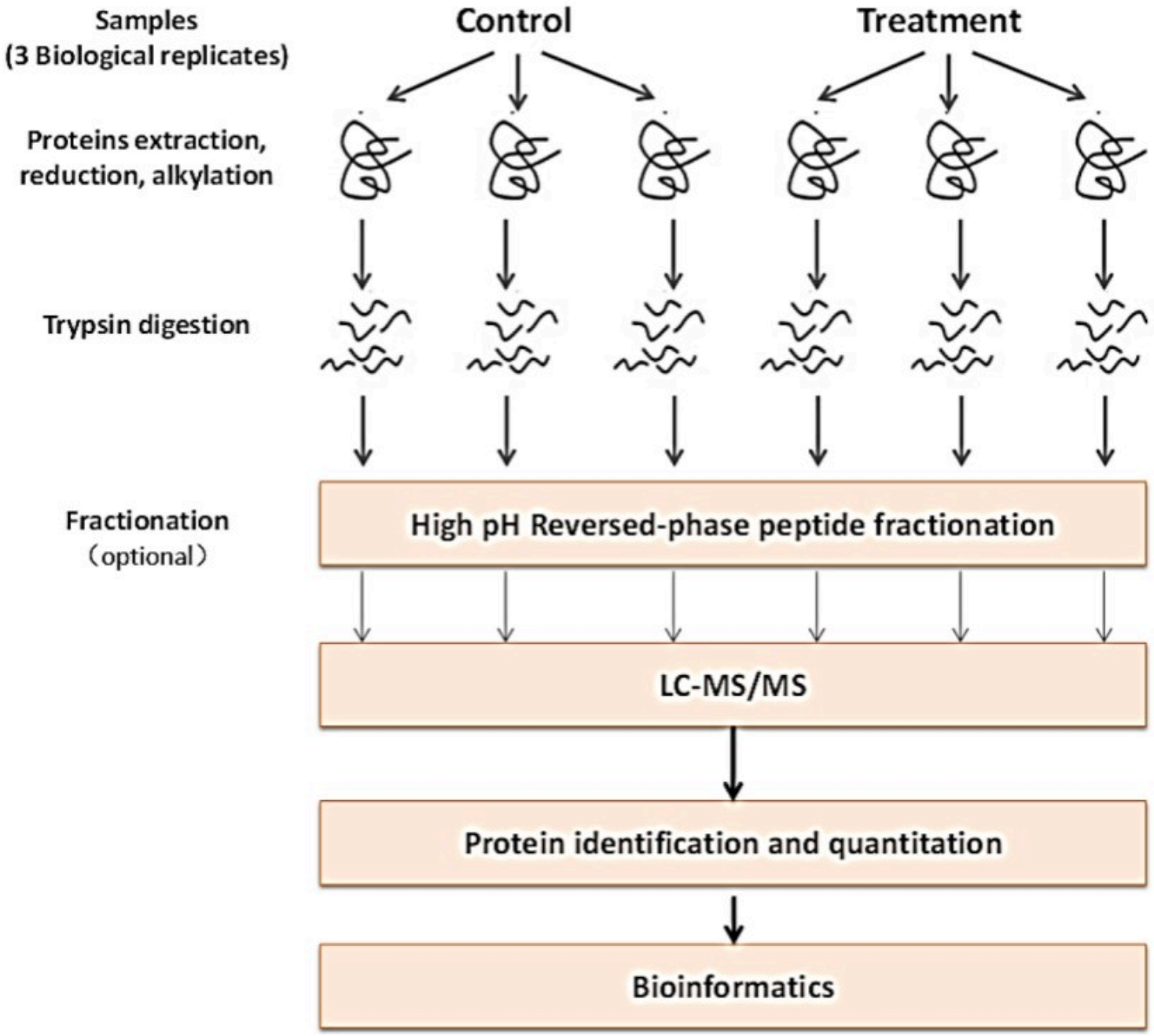


Figure 1

Flowchart of 4D-Label-free quantitative proteomics experiments.

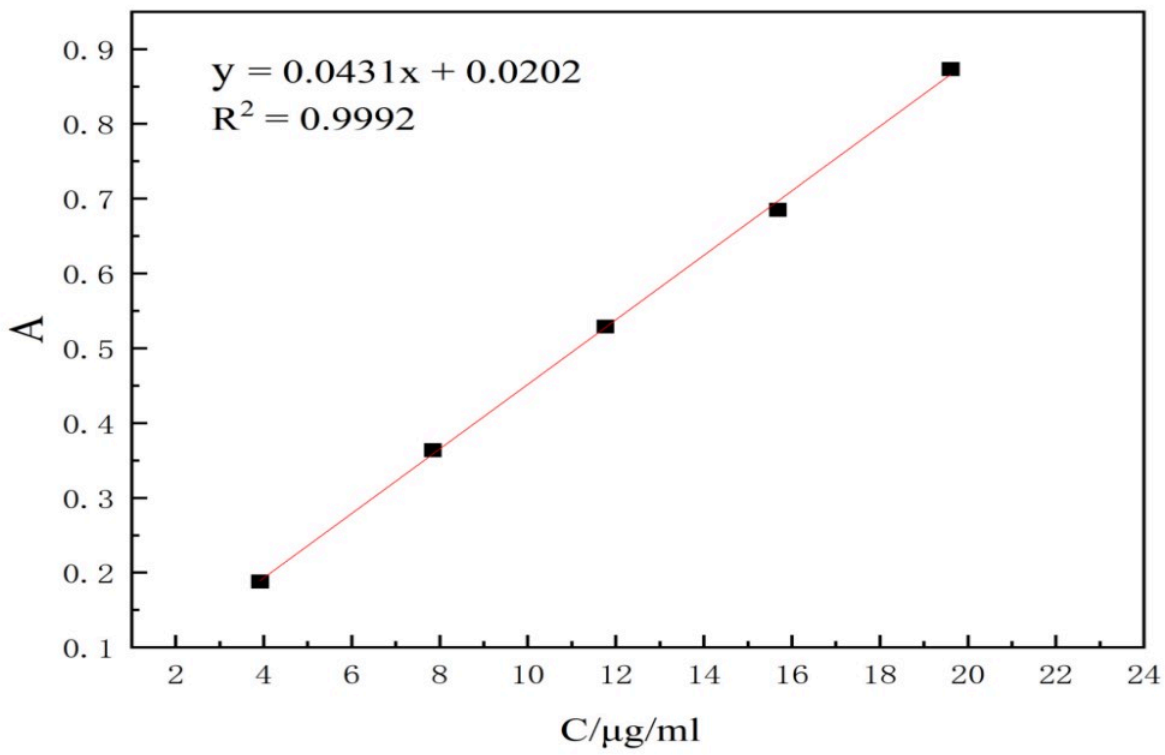


Figure 2

Protein standard curve.

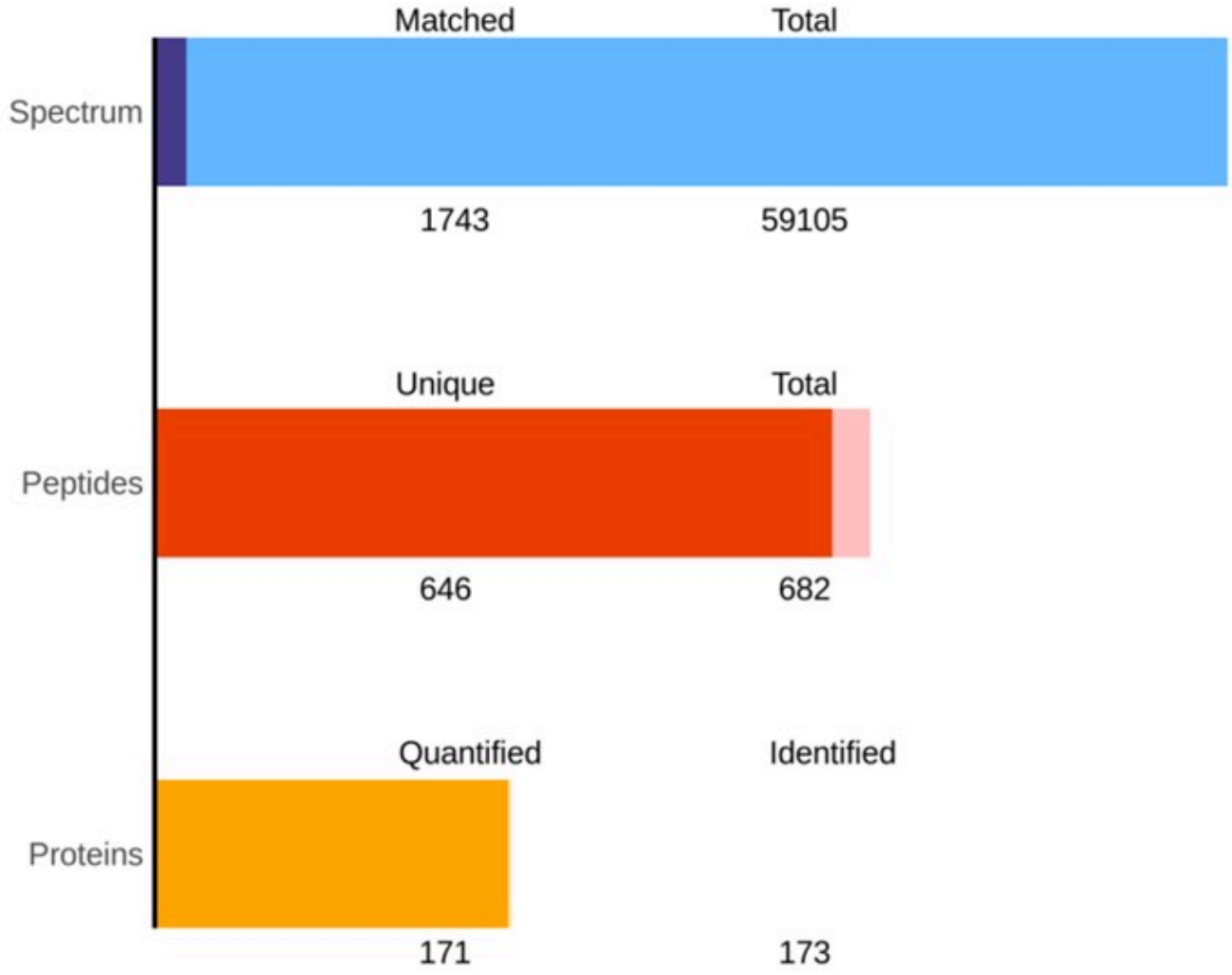


Figure 3

Identification and quantification results of the ProteinGroupA.

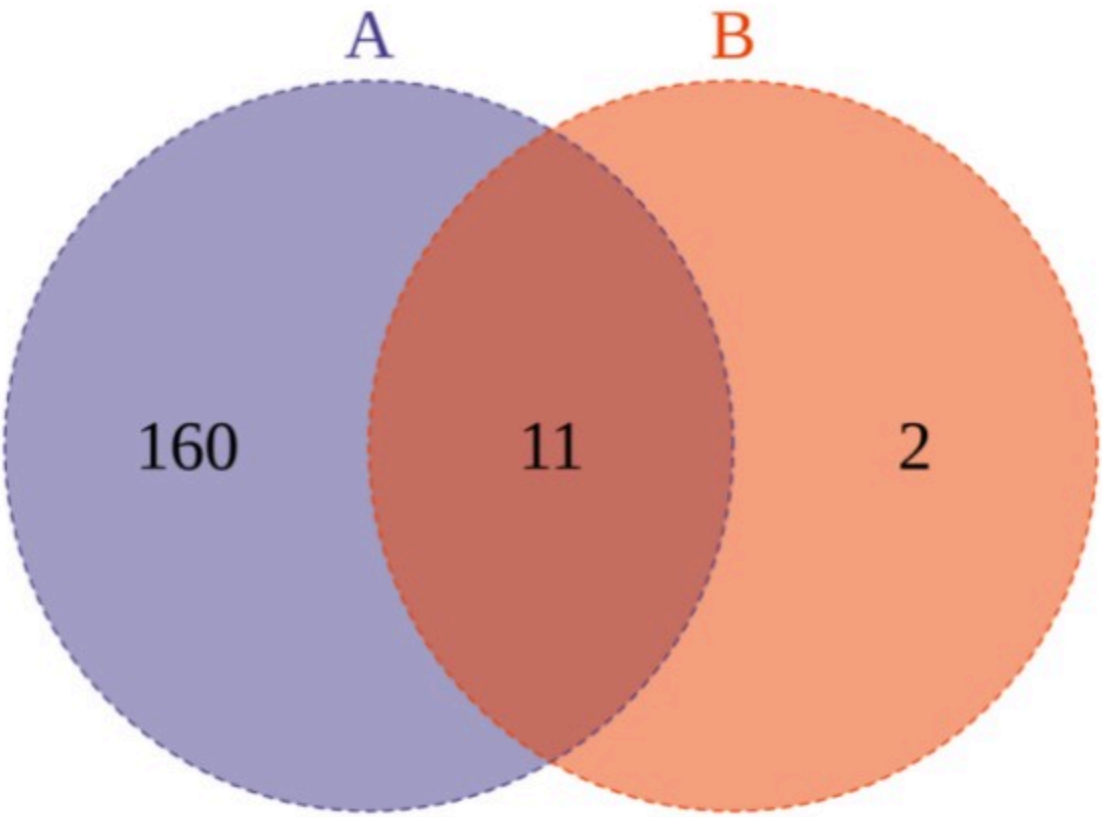
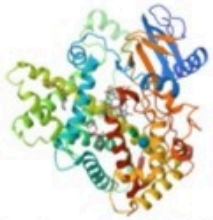
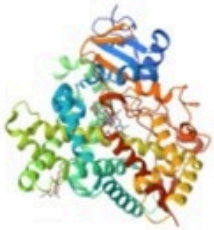


Figure 4

Venn diagram of proteins identified between all sample groups.



1.Cytochrome P450 CYP98A78 (3R1B)



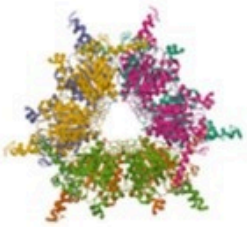
2.Cytochrome P450 CYP82D70 (3TMZ)



3.Enoyl-ACP ReductaseCYP82D70 (1UH5)



4.ABC Acyl Transporter (7VX8)



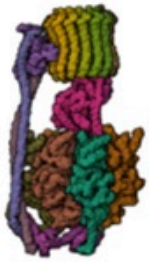
5.Carboxyltransferase beta subunit of Heteromeric ACCase (2A7S)



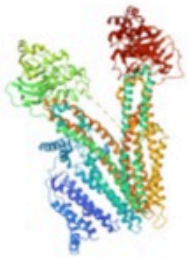
6.Universal stress protein 1 (1JMV)



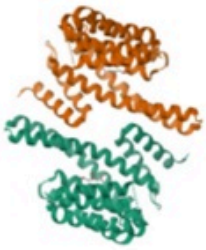
7.Universal stress protein 26 (6HCD)



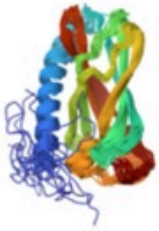
8.ATP synthase subunit beta, chloroplastic (6VM4)



9.ABC-type xenobiotic transporter (8HVH)



10.14-3-3-like protein C (Fragment) (1O9C)



11.Late embryogenesis-like protein (1YYC)

Figure 5

Conformations of the eleven same proteins (Database: PDB: https://www.rcsb.org/).

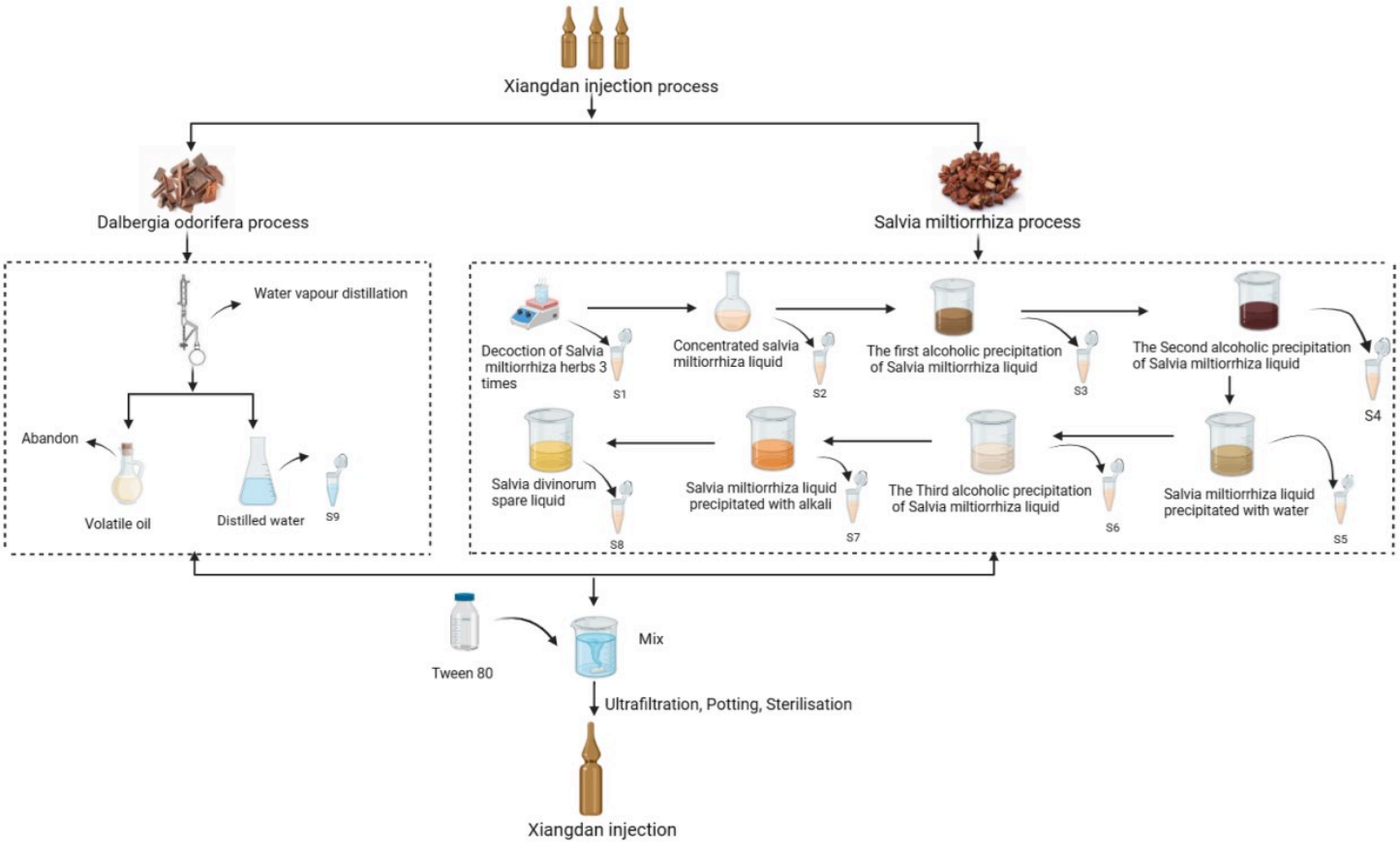


Figure 6

The whole process flow diagram of XDI and crucial steps of sampling points.

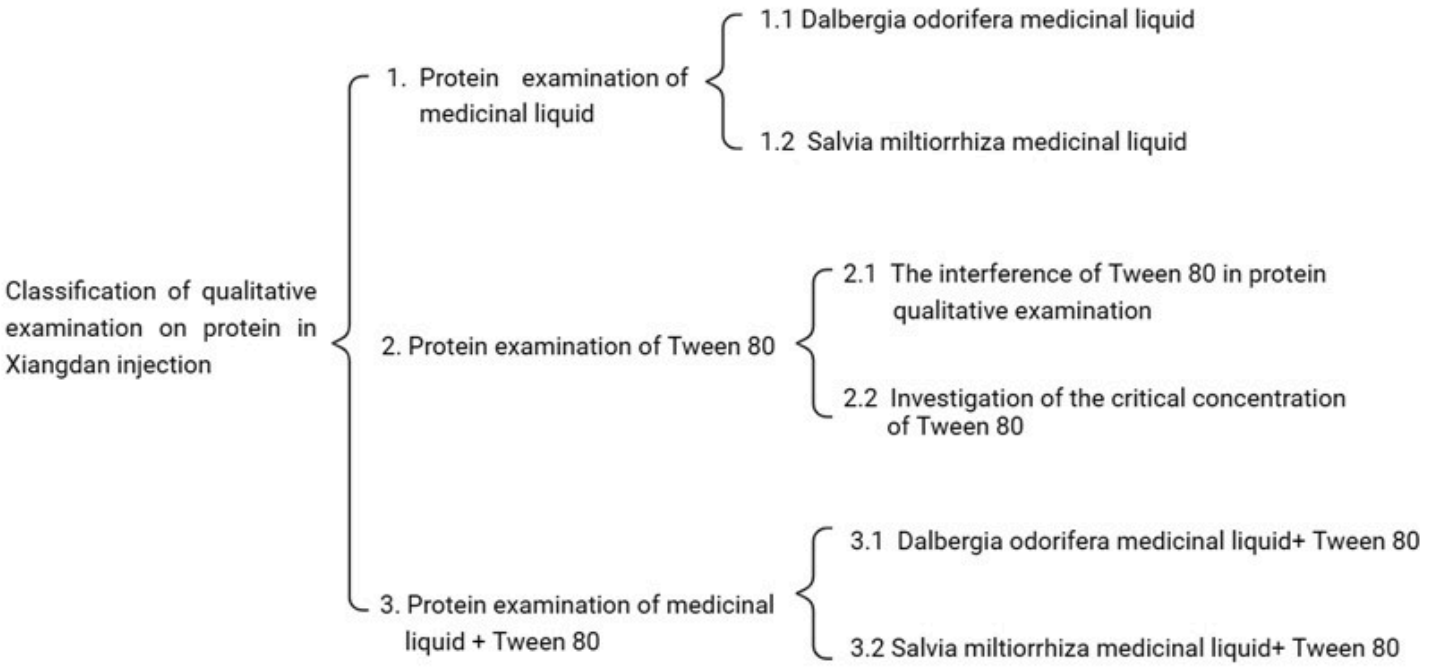


Figure 7

Three situations

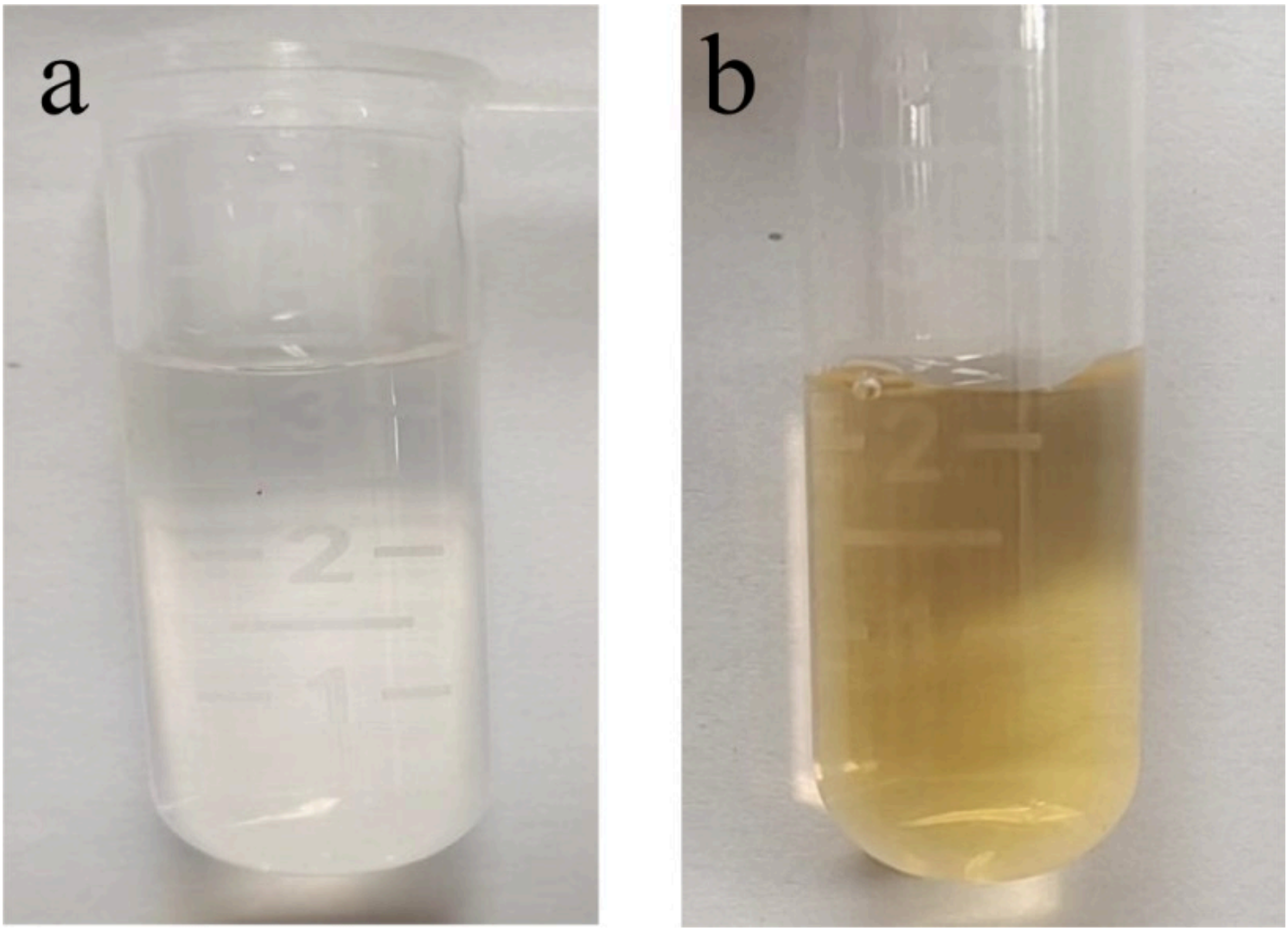


Figure 8

DO medicinal liquid did not precipitate with SA and TA. (a) SA+DO medicinal liquid; (b) TA+DO medicinal liquid

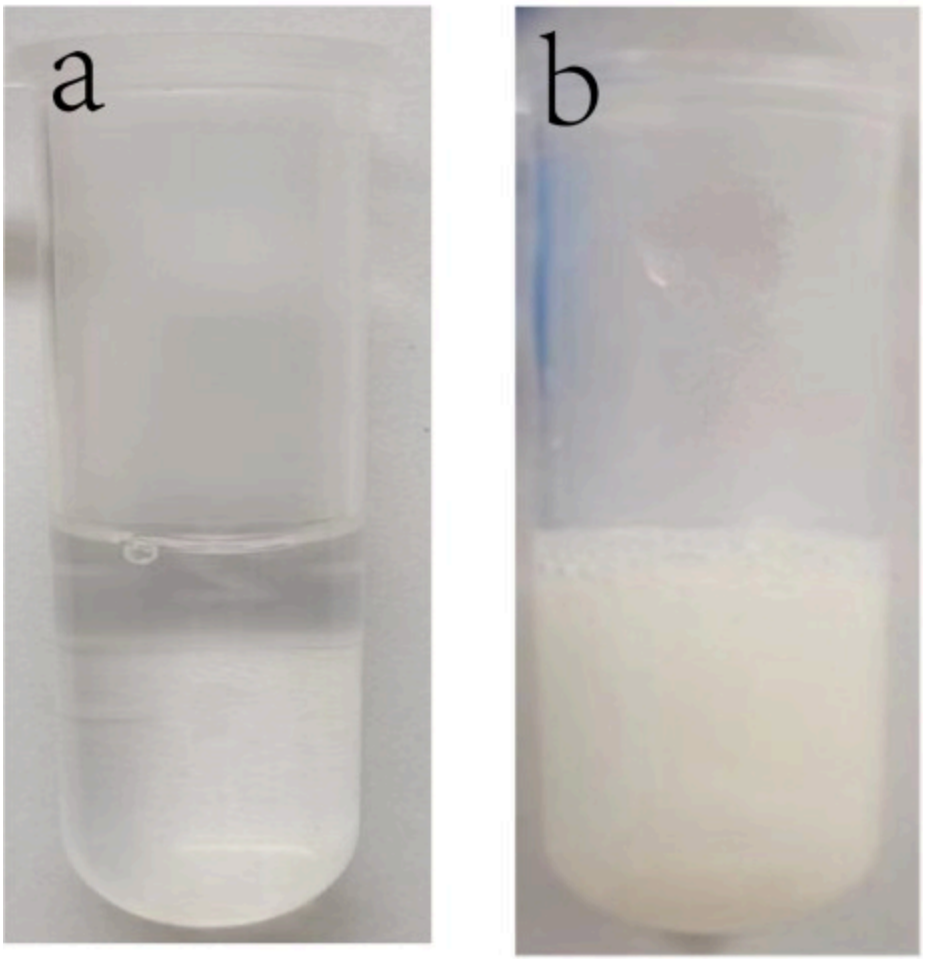


Figure 9

Qualitative examination results of DO medicinal liquid + Tween 80. (a) SA+DO medicinal liquid; (b) TA+DO medicinal liquid

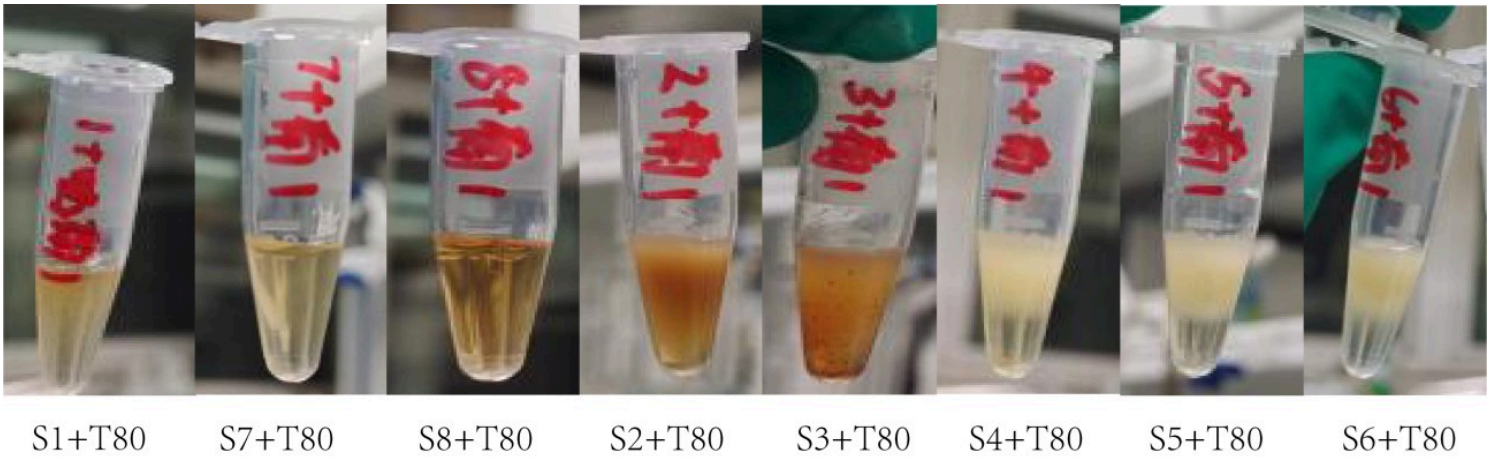


Figure 10

Qualitative examination results of the samples with SA.

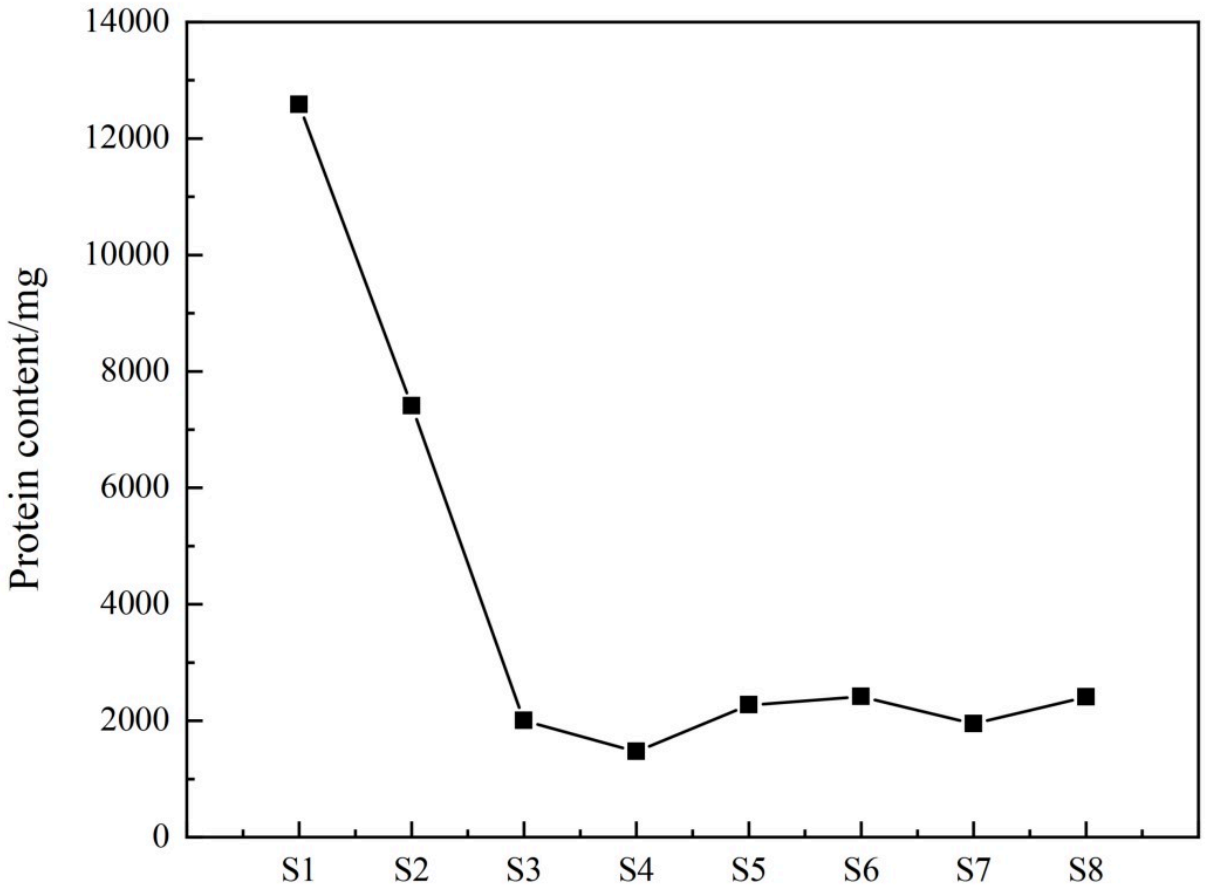


Figure 11

Variation of protein content in the production process of XDI

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.docx