Research progress on analysis methods of procyanidins

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Abstract. Procyanidins (PC for short) is a very complex secondary metabolite of plant polyphenols, rich in grape skins, seeds, apples, hawthorn, tea and other plants. Studies have shown that the intake of procyanidins can regulate cell apoptosis through its anti-oxidation effect, and play a role in maintaining body health, delaying aging and preventing diseases. However, there is no unified standard for detection and quantitative analysis methods so far, making the separation, purification, and characterization of such products always be a technical problem that plagues market quality evaluation. Therefore, this article summarizes and summarizes PC analysis methods, including spectrophotometry, high performance liquid chromatography and other analysis methods.

1. Introduction
Procyanidins (PC for short) generally refer to substances that can generate anthocyanidins under thermal acid treatment. This concept was first proposed by Weinges in 1960 [1], and has since been recognized by the academic community and is still used today. In earlier times, these complex plant polyphenols were usually classified as condensed tannins. Thompson gave a clear explanation of the chemical structure of PC [2] early on. He believed that these flavanol units were mainly connected by 4→8 or 4→6 carbon-carbon bonds, which polymerized into a very complex spatial structure. The basic unit structure of the polymer is shown in Fig. 1. The molecular size of procyanidins is determined by its degree of polymerization, usually oligomers with a degree of polymerization of 2 to 4, polymers with a degree of polymerization of 5 or more, and polymers with a degree of polymerization of more than 10, some polymers have a degree of polymerization even more than several hundred [3], its general structure is shown in Fig. 2.

![Figure 1. Structural unit diagram of procyanidin](image-url)
Studies have found that PC-based ingredients have a series of important health-care physiological functions such as superior anti-oxidation and scavenging free radicals. In recent years, they have been used as mature products in the plant extract market and are widely used as raw materials in health food, functional cosmetics and pharmaceutical industries.

However, although China has become the world's largest PC extract manufacturing base and one of the most important consumer markets for PC terminal products, it still faces a very difficult technical problem in terms of PC quality evaluation and quality monitoring, That is, reliable detection of the content of active ingredients in PC products and objective evaluation of the average degree of polymerization of PC.

2. Spectrophotometry

2.1. Bate-smith method

The carbon-carbon bond between the PC structural units will be broken in an acidic heating environment. The flavanol unit at the bottom will produce a flavanol molecule, while the remaining extension units at the top will first form unstable carbanions. And then oxidized to produce red anthocyanins [2]. Based on the characteristic of PC acid-catalyzed cracking, Bate-Smith explored and optimized the reaction conditions, replacing ethanol with n-butanol, which significantly reduced the speed of the entire side reaction, and then developed and established a spectrophotometric method to determine PC content [4].

This method usually uses the selected PC as a reference standard. Under an acidic heating environment, the absorbance of the reaction solution at 546 nm is measured to form a set of "procyanidin index", and then the actual sample is analyzed. Substitute the measured data into the corresponding formula to obtain the procyanidin index of the specific sample, which is then equivalent to the relative content of PC. This method is simple to operate and does not require complicated instruments, so it is still used today.

However, the PC content determined by this method is only a relative content, and sometimes the content exceeds 100, which is not the true percentage of PC. Dong [5] used a spectrophotometer to determine the content of anthocyanins in combination with the characteristic of PC to anthocyanin. At the same time, they strictly controlled the selection of standards, sample concentration and time and other factors, and determined Analytical method for determination of PC content by alcohol method. This method improves the accuracy of the determination results. According to reports, the relative...
standard deviation can be controlled within 0.32%~0.79%, the linear range is 0~200 μg/mL, and the spike recovery rate is 97.6~98.8%.

2.2. Porter method
The most significant defect of the above-mentioned Bate-Smith method is the uncertainty in the process of carbocation generating anthocyanins. The entire reaction process will be affected by many factors, making the reproducibility of the measurement results poor. Theoretically, the reaction of PC to produce anthocyanidins is an oxidation reaction, so the presence of metal ions will play an important role in the progress of the main reaction. Porter proposed that Fe$^{3+}$ ions played a certain catalytic role in the formation of anthocyanins. Under this premise, the Bate-Smith method was adjusted and 0.20 mL ferric ammonium sulfate solution was added to the reaction system. The results showed that the metal ions improve the sensitivity and precision of the results [6].

In addition, studies have shown that the internal water content of the system will also cause differences in results. In response to this situation, Porter proposed a water content control parameter, which is about 6%. The measurement result of the Porter method is expressed in the value of PVU (Porter value unit), which is similar to the calculation formula of the Bate-Smith method. The difference is that the latter supplements the dilution volume of 0.20 mL, so the dilution factor is changed from 7 to 7.2 before. The pigment yield has increased significantly, so the PVU value measured by the Porter method of the same sample is 3 times the Bate-Smith method procyanidin index, which greatly improves the sensitivity of the determination. Wang [7] used the Porter method to determine the PC content in camellia oil, 15 mL of a mixture of n-butanol and hydrochloric acid (95:5), then added 0.2 mL of 2% ferric ammonium sulfate solution, heated for 40 minutes, at 545nm for the determination of absorbance, the PC content was 265.90 mg/kg. Yan [8] found that there are interference components in the determination of PC in Haijiaoyan tablets by using the iron salt catalytic colorimetric method by adding the standard recovery method, respectively, using macroporous resin columns and ethanol precipitation-centrifugation methods to pre-purify the samples.

Finally, it is concluded that the ethanol precipitation-centrifugation method can significantly remove interference components and improve the accuracy of PC content determination. This method is a better sample pretreatment method.

2.3. Vanillin method
Under acidic conditions, phloroglucinol and resorcinol can condense with vanillin to produce carbanion compounds, which are colored substances. The flavanol monomer and its polymer contain phloroglucinol structure, which can also undergo condensation reaction with vanillin, and the content of flavanol is positively correlated with the color of the product. Therefore, a spectrophotometric method was constructed based on this principle.

However, the PC content obtained by this method will be higher than the actual content, but the accuracy is significantly better than the Porter method. Wang [9] established a vanillin-hydrochloric acid method for the determination of PC content in purple yam, 1 mL of purple yam extract, 5 mL of 10 g/100 mL vanillin solution, 3 mL of concentrated hydrochloric acid, and constant volume with methanol To 10 mL, let it stand at 20°C for 10 minutes, and measure its absorbance at 500 nm.

Song [10] used the vanillin-hydrochloric acid method to determine the PC content in different varieties of peanut coats, and determined the PC components in different varieties of peanut coats by HPLC, and found that the PC content and types of different varieties of peanut coats are different. Wang [11] based on the properties of vanillin reacting with sorghum tannins to form a more stable red compound, determined the content of sorghum tannins by spectrophotometry, and at the same time improved the catechins that are often used as the vanillin method. Quantitative analysis of PC was carried out and the reaction conditions were optimized, and the measurement results were closer to the true content of tannins in sorghum.
2.4. Folin-Ciocalteau method
Phenolic compounds have a reducing effect in an alkaline environment, which can reduce $W^{6+}$ in tungstomolybdic acid to $W^{5+}$. The color of the resulting compound is blue, and the color is positively correlated with the content of polyphenols. Generally, there is an absorption maximum at 760 nm, and gallic acid is often used as a reference for quantitative research. This method has high sensitivity and easy operation, but it has certain defects in specificity. Many reducing substances coexisting in natural products, including phenolic components, proteins, ascorbic acid, nucleic acids, etc., can all react in this way, causing positive errors in the analysis results.

3. High performance liquid chromatography(HPLC)
The HPLC method has excellent separation and quantitative analysis performance. Among a variety of detectors, especially ultraviolet and diode array detectors, it is very suitable for the analysis and detection of phenolic substances. The application of HPLC method in PC analysis is mainly reflected in the following aspects.

3.1. Direct HPLC method
To put it simply, it is to directly determine the components of PC. At present, there are abundant research reports in this area at home and abroad, mainly applied to grape seeds[12,13], apple[14-16], tea polyphenols[17-19] Waiting for PC content analysis. When applying this method, PC monomer reference substance or PC reference substance is usually used as an external standard to establish a quantitative analysis method for chromatographic peaks.

Huang [20] and others used methanol-acetonitrile-water as the mobile phase, and applied HPLC method to analyze the PC content of longan. The research results showed that many additional components of OPC and PPC are distributed around the main components, so it is necessary to select a relatively Ideal chromatographic peaks are more difficult. Svedström [20] used acetonitrile-water as the mobile phase to analyze the content of the European hawthorn OPC 2~6 polymer. Before the analysis, two column chromatography were required to pre-process the sample. The operation process is cumbersome and it is difficult to guarantee the results. Accuracy, and many important components such as catechins, epicatechins and flavonoids cannot be measured at the same time.

Theoretically, the spectrophotometric method can only select a certain type of control substance to determine the PC content, and the accuracy of the direct-HPLC method is higher than that of the spectrophotometric method, but the applicable monomer types are relatively limited, and it can only be used for children. Analysis of tea, epicatechin and some oligomers, such as B2, B1 and B5. In fact, the PC-type components are extremely complex. When performing direct-HPLC analysis, multiple peaks are superimposed on each other, messy and unrecognizable at all.

There are few PC standard products on the market, so the defects are very significant when applying this method. Not only that, combined with previous studies, we can see that the results obtained by this method are less reproducible [21, 22]. This is mainly due to the influence of the complex components of PC. Any small change in conditions, such as column temperature, mobile phase ratio, and chromatographic column selection, will have a significant impact on the separation, which in turn manifests as a deviation in the results.

3.2. Porter-HPLC method
This method processes the samples according to the Porter method and then uses HPLC for analysis. Procyanidin monomer is catalyzed by iron salt in an acidic heating environment to produce deep red anthocyanin ions, which are then analyzed by HPLC. When analyzing, it is necessary to pay attention to the control of retention time, use time as a variable, and perform quantitative research in combination with external standard method. At present, this method has been recognized by researchers as the "Determination of Proanthocyanidins in Health Foods” (GB/T 22244-2008).

Compared with the Porter method, this type of method is based on the product anthocyanin for the measurement, which effectively eliminates the measurement effects of other colored substances, and
shows a greater advantage in the measurement results. It should be pointed out that the reference substance is not an anthocyanin, but a standard procyanidin. As a result, differences in plant sources and extraction methods will have a certain impact on the color level of procyanidins, especially the degree of polymerization of PC will directly affect the yield of anthocyanins. Therefore, it is necessary to select materials reasonably to ensure the overall improvement of the purity of the production of standard procyanidins.

It is necessary for researchers to conduct more in-depth discussions in this aspect. Song [23] determined the content of anthocyanins in blueberry beverages, and the content was 50.0 mg/g. It is worth emphasizing that this method only quantitatively analyzes the amount of procyanidin extension units, which does not include colorless flavanols, so it may cause errors in the final results to some extent.

3.3. Thiolysis-HPLC method

In the 1960s, some researchers carefully studied the interaction between flavonoids and thioglycolic acid. As a nucleophile, thioglycolic acid was added to acetic acid to react with benzyl alcohol and benzyl ether. Some people use hot butanol and hydrochloric acid to degrade procyanidins. The above researches are of great value for flavonoid tannins and the chemical structure of procyanidins.

Thompson [2] proposed that when procyanidins are in an acidic environment, if the temperature reaches an appropriate level, the structure of procyanidins will be broken, and the upper part of the fracture is a carbanion intermediate, which has poor stability. It reacts with nucleophiles to generate flavanol-benzyl sulfide derivatives, and the broken lower part generates flavanol monomers, as shown in Fig. 3.

![Figure 3. Procyanidin thiolysis reaction](image)

Therefore, with the help of this feature, a quantitative experiment was carried out. Only by analyzing the content of flavanol monomers and benzyl sulfide derivatives, the specific procyanidin polymer content can be grasped. By means of numerical comparison between the total content of thiolysis products and the content of flavanol monomer molecules, the average polymerization degree parameter of procyanidins can be obtained.

At present, based on this principle, an acid-hydrolyzed benzyl mercapタン derivatization-HPLC analysis method has been developed, and certain results have been achieved in practical applications [24-30]. As shown in Table 1. The HPLC analysis of grape seed extract before and after thiolysis reaction is shown in Fig. 4 and Fig. 5. It can be seen that the chromatogram of the grape seed extract is very simple after the thiolysis reaction, and the large peak of polymer impurities that originally gathered at 30–35 minutes has been basically eliminated, which makes the quantitative analysis of PC The result becomes more accurate and reliable.
Table 1. Application of thiolysis-HPLC method in recent years

| Researcher  | Material                  | Method              | Result                                                                 |
|------------|---------------------------|---------------------|------------------------------------------------------------------------|
| Ramsay[31] | Carambola fruits and leaves | Thiolysis method and LC-MS | Carambola fruits and leaves are mainly composed of B-type procyanidins |
| Ropiak[32] | Thirty European medicinal plants | Thiolysis method | The condensed tannin content, the average degree of polymerization, the ratio of procyanidin/prodelphidine and the ratio of cis/trans flavantriol were measured. It also showed that four samples were A-type junctions, such as blackthorn flowers, heather flowers, bilberry leaves and bilberry leaves. Only one sample contained gallicized procyanidins, such as Soil rhubarb. |
| Fryganas[29] | The herb of Red bean | Thiolysis method and NMR 13C spectrum | It is necessary to correct the procyanidin content. In the 6 samples of red bean grass, the content of procyanidins was 1.6 to 20.8 times higher than that after thiolysis, and 1.4 to 2.6 times higher than the hydrochloric acid-n-butanol-acetone test results. |
| Li[30] | Polyphenols in red wine | Thiolysis-HPLC method | Purity and average degree of polymerization of procyanidin polymer |
| Gao[33] | cranberries | Thiolysis-HPLC method | Quantitative analysis of the ratio of procyanidins to type A procyanidins, using cysteamine as a substitute for low-odor benzyl mercaptan to participate in the thiolysis reaction |
| Luo[34] | Grape seeds and grape skins | Thiolysis-HPLC method | Procyanidins and average degree of polymerization |

Figure 4. HPLC analysis of grape seed extract before thiolysis
Figure 5. HPLC analysis of grape seed extract after thiolysis

Currently, the thiolysis-HPLC method is widely used in the study of the procyanidin content of grape, apple, hawthorn and other materials. The thiolysis reaction methods and chromatographic analysis conditions reported in different literatures are slightly different. But the operation link is relatively simple. On the one hand, this method can accurately perform qualitative analysis, on the other hand, it also provides technical support for quantitative research, and it can also provide important information about mDP. Therefore, it will play an important role in the analysis and research of procyanidins in the future. However, even so, this method still has certain quantitative analysis flaws. First, the application of this method is based on the premise of complete reaction. However, in fact, the concentration of benzyl mercaptan and the ratio of added acid will affect the thiolysis reaction, and it is difficult to achieve complete degradation. Second, ignoring the difference in relative molar absorption of thiolysis products, it is believed that EC-S can be quantified by EC. Finally, it is directly recognized that the synchronization and stability of EC and EC-S exist. However, in the actual reaction process, both types of substances will be degraded to a certain extent [35]. Such situations greatly reduce the accuracy of quantitative analysis. It may not be easy to obtain standard samples of thiolysis reaction products. At present, there is no research report on the preservation rate of reaction products in thiolysis reaction system.

4. Auxiliary technology for HPLC analysis
Due to the complexity of the structure of procyanidins, it is often difficult to obtain accurate measurement results according to conventional detection methods, such as HPLC. Therefore, experts and scholars have made unremitting efforts to develop a series of sample pretreatment techniques, such as coagulation. Gel permeation chromatography technology, silica gel column chromatography, Toyopeah TSKHW40, etc. With the support of this type of technology, procyanidins can be effectively separated, which greatly improves the accuracy of quantitative analysis, but these methods also have potential for many experimental errors. As far as chromatographic analysis is concerned, it is usually a reverse phase chromatographic gradient elution procedure, and the eluent is methanol or acetonitrile.

In terms of detectors, array diode detectors in tandem mass spectrometry detectors are often used, such as LC-MS with electrospray ion source. This method can be used for more reliable qualitative analysis of chromatographic peaks. However, due to the limited range of mass-to-charge ratios, this
method monitors oligomers composed of up to 6 catechin (CA) units. Therefore, it is necessary to use multi-charged ion detection for higher quality polymer research and determination.

5. Other analysis methods

5.1. Capillary electrophoresis
The quartz capillary tube is used to construct the corresponding separation pipeline, and then under the influence of the direct current electric field, each element in the sample is promoted to reflect the differentiated channel distribution characteristics, so as to realize the separation between the components. Li [36] conducted an in-depth analysis of the separation environment and conditions of capillary electrophoresis, and concluded that the separation of procyanidins can achieve good results when in a pH 9 borax phosphate buffer system. The borate buffer causes the separation effect of many procyanidin isomers and procyanidins with different degrees of polymerization, which in turn promotes the continuous movement of electric particles in a plunger shape, so as to obtain the corresponding electrophoretic pattern. This method requires less capital investment, is easy to control, and has a short duration, but it has certain disadvantages in terms of reproducibility.

5.2. Spectrally sensitive pulse photometry
Some researchers reacted wine with protein substances to form procyanidin-protein complexes in water. Optical equipment working with wavelength-sensitive pulsed electromagnetic sources was used to combine the different intensities and spectral emission of the light source, and the reaction solution was optically processed at room temperature. Measurement, and then get the relationship between the content of procyanidin and the turbidity of the reaction solution. This method is simple and cost-effective, and provides a reliable alternative to time-consuming analysis [37].

5.3. Atomic absorption spectrophotometry
From the principle of the method, it is difficult to measure procyanidins. The measurement mode is to determine the specific procyanidin content by correlating procyanidins and metal ions, and then measuring the amount of metal ions remaining in the solution. Ma [38] put procyanidins in a neutral solution and carried out a related chemical reaction with lead ions, resulting in a brown-yellow precipitate. After further filtering the precipitate, the filtrate Pb\(^{2+}\) was measured, and then the Pb\(^{2+}\) proportion of similar substances indirectly derives the content of procyanidins. However, the linear range of the standard curve of this kind of method is relatively small, and it is difficult to guarantee the accuracy of the measurement result when the sample structure is complicated.

In addition to the above methods, researchers have chemically stained procyanidins with 4-dimethylaminocinnamaldehyde, and identified the procyanidin location of pear pulp at the mature and mature stage through optical microscope and projection electron microscope [39]. In addition, there are physical detection methods such as infrared spectroscopy. At present, some scholars have used this method to study the chemical structure of black rice procyanidins [40], and there are also methods used and practiced around the procyanidin content of larch bark [41]. However, there are usually other coexisting organic substances in the sample, which makes the analysis of infrared spectroscopy face many obstacles. Compared with the former, the quantitative nuclear magnetic resonance (qNMR) method is a more efficient physical detection method [42].

This type of method can analyze the sample content and concentration information without material comparison, and conduct a comprehensive Intuitive display. The \(^1\)H-NMR signal shows certain advantages in quantitative analysis; the \(^{13}\)C-NMR signal reflects a higher level of chemical shift resolution, which is particularly effective in qualitative detection. Combined with the selection of reasonable monitoring peaks, a systematic analysis of sample isomers can be carried out. However, the reason why this type of method is not widely used at present is mainly because of the large amount of capital investment in testing instruments and the high cost, which is difficult to afford for many ordinary laboratories.
6. Conclusion

In the field of health products, my country has always used some insufﬁciently rigorous ingredients such as "polyphenols", "total ﬂavonoids" and "total extracts". If there is no corresponding accurate analysis and testing methods, market supervision will be at risk. Establishing a complete product quality evaluation system is one of the basic conditions to ensure the normal operation of the modern social commodity economy. However, there are still some challenging analytical problems that have not been overcome for a long time, such as the analysis of natural products such as procyanidins and ﬂavonoids. Although after 50 years of continuous exploration and research, many studies have been made on the qualitative and quantitative analysis of procyanidins. As a result, many qualitative and quantitative research methods suitable for different purposes have been constructed. However, the current high-eﬃciency determination methods for procyanidin content require further in-depth research, and many areas still need to be improved.

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