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Abstract: This paper discusses a new method for determining the iron content in Chinese fir leaves and roots using a SmartChem200 automatic chemical analyzer. With a pH value of 4-5, Fe^{3+} was reduced to Fe^{2+} by hydroxylamine hydrochloride, and Fe^{2+} and 1,10-phenanthrolinemonohydrate generated an orange complex. The iron content was calculated based on the absorbance measured at a wavelength of 510 nm. The absorbance is directly proportional to the Fe^{2+} content within a certain concentration range of Fe^{2+}. The absorbance had a good linear relationship with the Fe^{2+} content in the range of 0.01 $mg \cdot L^{-1}$ to 5 $mg \cdot L^{-1}$; the linear regression equation of the standard curve was Y=0.0632**X*-0.00002, and the correlation coefficient was 1.0000. The method is convenient and rapid, and the experimental results were reliable.

1. Introduction

Currently, the general methods for iron content determination are usually spectrophotometry (Biqiong and Hua, 2013), atomic absorption spectroscopy (AAS) (Pereira et al., 2014, Khan and Cornfield, 1968), and inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Yue, 2014). Spectrophotometry is generally low cost, and daily maintenance of the instrument is convenient; however, the methods require complex operation and many steps, and there is increased equipment and human error while determining iron content by this method (Lysionek et al., 1998). In addition, there is low repeatability, poor stability, and a narrow detection concentration range, and the chromogenic reaction is influenced by temperature, pH and medium conditions. The AAS method to determine the iron content of a plant utilizes a high temperature graphite furnace, and although this method has a high analysis speed, it is difficult to achieve a satisfactory standard of quantitative and linear analysis based on the AAS principle and on practical tests of the existing equipment ($r \geq 0.99$). The ICP-AES method is very fast and it can determine a variety of elements at the same time, but more expensive instruments are needed in addition to daily maintenance. Therefore, it is important to explore a new method for determining iron content that has the benefits of speed and low cost. The SmartChem200 automatic chemical analyzer uses a colorimetric analysis of the micro-response, and with its high degree of automation and fast analysis speed, it can measure more than 200 samples

within an hour. It has been widely applied for determining indicators such as ammonia nitrogen, phosphate, nitrate, and nitrite in water quality, soil, tobacco, plants, and other fields. The use of a flow injection method to detect iron content has previously been reported (de Oliveira et al., 2014, AMR et al., 1998, Yebra, 2012, Sahin et al., 2010, Kass and Ivaska, 2002), but using a SmartChem200 automatic chemical analyzer to detect the iron content in plants has not been reported. In this article, we describe a new determination method for iron content in the roots and leaves of Chinese fir using a SmartChem200 automatic chemical analyzer, which showed good success and results with higher accuracy and repeatability. Furthermore, this method effectively reduces the labor intensity and is particularly suitable for determining the iron content in a large number of samples. Because of the use of micro-reactions, the chemical reagents and samples are only used in microliter volumes, and therefore, the process results in a simultaneous reduction in cost and secondary pollution of the laboratory.

2. Materials and Methods

2.1. Plant materials and growth conditions

Chinese fir seedlings were provided by the State Forestry Administration Engineering Research Center for Chinese Fir. Short, 2 cm shoots were initially grown in basic MS (Murashige and Skoog) medium, pH 5.8, containing 0.25 $mg \cdot L^{-1}$ IBA and 0.33 $mg \cdot L^{-1}$ 6-BA and supplemented with 4% sucrose and 0.6% agar. After 60 d, the short, 4 cm shoots were shifted to a rooting medium for plantlet regeneration (1/4 MS medium, pH 5.4, containing 0.14 $mg \cdot L^{-1}$ IBA and 0.075 $mg \cdot L^{-1}$ NAA and supplemented with 2% sucrose and 0.65% carrageenan), placed in the dark for 7 d at 25±3 °C, and then transferred to the light at 25±3 °C. The photoperiod was 12 h (8:00-20:00), and the light intensity was 2000 lux.

2.2. Aluminum treatment

After the seedlings had grown for 30 d in the rooting medium, seedlings with similar initial root lengths were washed with distilled water, inserted through a foam support plate and transferred to a plastic container filled with Hoagland-Arnon solution (0.51 $g \cdot L^{-1}$ KNO_3, 0.136 $g \cdot L^{-1}$ KH_2PO_4, 0.49 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.82 $g \cdot L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 0.0139 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.01865 $g \cdot L^{-1}$ EDTA-Na_2, 2.86 $mg \cdot L^{-1}$ H_3BO_3, 0.09304 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, pH 4.0) containing 0, 0.5, 1, 2 or 4 $mmol \cdot L^{-1}$ $AlCl_3$. The aluminum stress experiment was conducted in a controlled growth chamber at 25 °C with a 12 h light:12 h dark photoperiod, at 60% constant relative humidity, and at a light intensity of 2000 lux during the day. Each treatment was repeated three times with three biological replicates, and each replicate consisted of 30 seedlings. After 1, 4, 8, 16, 32 and 64 h at different aluminum stress levels, the ground and underground parts of five plants seedlings in each replicate were sampled separately. The samples were dried at 105℃ for 15 min, followed by drying at 65℃ to achieve a constant weight, and were finally placed in 5 ml tubes; then, two 5 mm sterilization steel balls were added into the same tube. The tubes containing the dried samples were then shaken in a high-throughput tissuelyser (Scientz-192, Xinzhi, Ningbo) for 3 min at a frequency of 20 (1200 times/min) at room temperature.

Precisely weighed 0.2 g ground samples were placed in 50 ml conical flasks, a small amount of water (approximately 3 ml) was added to wet the samples to be tested, and 20 ml of mixed acid (HNO_3:$HClO_4$=5:1, V:V) was then added through a small funnel in the top of the bottle. The samples were then boiled using a Labtech digital display electric heating plate (EG35B, Labtech, Beijing, China), and the temperature was controlled within 300℃ to keep the digested liquid mildly boiling. When no brown gas was emitted and only some white water vapor was emitted in the digestion bottle, the digestion was finished. The cooled digested liquids were filtered through a 50 ml volumetric flask with quantitative filter paper and later transferred to 60 ml small white plastic bottles until ready for testing. The blank solutions were prepared using the same process.

2.3. The compilation of the method

2.3.1. The principle of the method At a pH value of 4-5, Fe^{3+} was reduced to Fe^{2+} by hydroxylamine hydrochloride ($NH_2OH \cdot HCl$, sigma), and Fe^{2+} and 1,10-phenanthrolinemonohydrate ($C_{12}H_8N_2 \cdot H_2O$, Shanghai) generated an orange complex. The iron content was calculated based on the absorbance measured at a wavelength of 510 nm.

2.3.2. The instrument operation steps To begin testing, the first step is to open the computer and the automatic chemical analyzer (Smartchem200, Italy) and then enter the workstation software from Smartchem200. We then preheated, cleaned and conducted the water base line (WBL) measurement according to the daily operating procedures (the next step can be performed while all of these are running). Next, create the test plan by entering the sample number, selecting the test method, and then according to the sample worksheet markers, place the sample under the test with distilled water and the standard solution and reaction reagent. The settings of the measurement conditions and the concentration of the work curve is located in the workstations software setup. The measurement condition mainly inspects the quantity of samples, including the addition of the volume of Reagent3 (FLA), Reagent4 (FLB) and Reagent5 (FLC), and the reaction time. Sample quantity can be adjusted between 1 μl and 300 μl based on the size of the sample concentration, and the sample quantity can be reduced when the iron concentration of the sample is high. However, the sample quantity can be increased when the iron concentration of the sample is low, so that the absorbance can be adjusted to the proper range to effectively improve the accuracy of determination results; but the sample quantity cannot be more than 300 μl. In addition, the volume or the concentration of Reagent3, Reagent4 and Reagent5 can also be adjusted, but the total volume of the sample, Reagent3, Reagent4, Reagent5 and rinse solution should not be more than 679 μl, and the total volume of the sample and Reagent3 should be in the range of 290-390 μl. The settings of the work curve concentration: the Smartchem200 automatic chemical analyzer has a function that permits automatic dilution of the sample liquid while entering the corresponding dilution multiple in the method operation interface, and the required series of standard solutions can be acquired when the standard stock solution and the corresponding reaction reagent is placed in the instrument; the reagent can be automatically diluted in the testing process, followed by the automatic analysis of the measured results.

2.3.3. The reagents used in the iron method Smartchem200 cleaning solution: 30 g potassium hydroxide (KOH, Sinopharm), 36.3 g Tris ($C_4H_{11}NO_3$, sigma), 32 ml Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$, sigma), 8 ml isopropanol (C_3H_8O, Sinopharm), and distilled water to a volume of 1000 ml.

Smartchem200 rinse solution: 25 g Brij35 polyethylene glycol monooleyl ether ($C_{38}H_{76}O_{11}$, sigma) dissolved in distilled water, with distilled water added up to a final volume of 100 ml.

Reagent 1 (rinse solution): add 1 ml Smartchem200 rinse solution and 2000 ml of distilled water to the bottle of rinse solution.

Reagent 2 (cuvette cleaning solution): add 100 ml Smartchem200 cleaning solution and 1900 ml of distilled water to the bottle of cleaning solution.

Reagent 3 (FLA): 0.1 ml Smartchem200 rinse solution was dissolved in distilled water, and distilled water was added to a volume of 100 ml.

Reagent 4 (FLB): 20 g sodium acetate (CH_3COONa, Sinopharm) and 2 g hydroxylamine hydrochloride ($HO\text{-}NH_2 \cdot HCl$, Sinopharm) were dissolved in 90 ml of distilled water using acetic acid (CH_3COOH, Sinopharm) to adjust the pH value in the range of 4 and 5. Finally, distilled water was added to a volume of 100 ml.

Reagent 5 (FLC): 0.2 g 1,10-phenanthrolinemonohydrate ($C_{12}H_8N_2 \cdot H_2O$, Sinopharm) was dissolved in 60 ml of distilled water, and distilled water was added up to a final volume of 100 ml (fresh preparation, the remaining solution was kept in a refrigerator and was effective for up to one month).

Standard solution (5 $mg \cdot L^{-1}$): 0.5 ml of 1000 $mg \cdot L^{-1}$ iron standard solution (National Center of

Analysis and Testing for Nonferrous Metals and Electronic Materials: GSB 04-1726-2004) was added to distilled water to a produce dilution with a constant volume of 100 ml.

2.4. The calculated results

2.4.1. The formula for iron content $W_{Fe} = \frac{c \times V \times t_s}{m \times 10^6} \times 1000$

W_{Fe}: iron content ($g \cdot kg^{-1}$); c: the concentration of iron was obtained from the working curve ($mg \cdot L^{-1}$); V: the volume of the coloring solution (50 ml); m: the weight of the dried samples (g); t_s: divide ratio $t_s = \frac{\text{the volume of boiling liquid tested（ml）}}{\text{the volume of test liquid absorbed in the test（ml）}}$.

2.4.2. The calculated recovery rate of the standard addition $P = \frac{(C2 - C1)}{C3} \times 100\%$

P: the recovery rate of the standard addition (%); C1: the measured specimen concentration ($mg \cdot L^{-1}$), C1 =m1/V1; C2: the measured concentration of the standard addition specimen ($mg \cdot L^{-1}$), C2 =m2/V2; C3: the added standard ($mg \cdot L^{-1}$), C3 =C0×V0/V2; m=C0×V0; m1: the content of the specimen (g); m2: the content of the standard addition specimen (g); m: the content of the standard addition volume (g); V1: the volume of the specimen (ml); V2: the volume of the standard addition specimen (ml), V2=V1+V0; V0: the volume of the standard addition (ml); C0: the standard solution concentration of the standard addition ($mg \cdot L^{-1}$). In this article, C0=50 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$, 200 $mg \cdot L^{-1}$; V1=4.95 ml; V0=0.05 ml; and C3=0.5 $mg \cdot L^{-1}$, 1 $mg \cdot L^{-1}$, 2 $mg \cdot L^{-1}$ were used to calculate the recovery rate of the standard addition.

3. Results and Discussion

3.1. Absorption spectrum

To determine that the experimental results had a higher sensitivity, the maximum absorption wavelength was needed to determine the absorbance value of the solution. According to the amount of several reagents, which are listed in Test Number 1 in Tab.1, and the final added iron standard concentrations of 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 5.0 $mg \cdot L^{-1}$ were scanned in the spectral range from 400 nm to 900 nm. The results showed that the maximum absorption wavelength of the orange complex generated by Fe^{2+} and 1,10-phenanthrolinemonohydrate, is located at 510 nm (Fig. 1).

Tab.1 The screening of measurement conditions

Test Number	Sample Quantity /μl	FLA/μl	FLB/μl	FLC/μl	Read Time/s	Correlation Coefficient (R^2)	Method Name	Note
1	200	180	130	100	684	0.9999	FEA1	Normal
2	200	180	130	100	558	0.9988	FEA2	High
3	200	180	130	100	396	0.9998	FEA3	High
4	200	180	130	100	216	0.9999	FEA4	High
5	200	180	130	100	108	0.9998	FEA5	High
6	200	180	130	100	36	0.9999	FEA6	High
7	180	180	130	100	684	0.9999	FEB1	High
8	160	180	130	100	684	0.9999	FEB2	High
9	140	180	130	100	684	0.9997	FEB3	High
10	120	180	130	100	684	0.9999	FEB4	High
11	100	180	130	100	684	0.9996	FEB5	High
12	200	160	130	100	684	0.9999	FEC1	High
13	200	140	130	100	684	0.9995	FEC2	High
14	200	120	130	100	684	0.9997	FEC3	High

15	200	180	110	100	684	1.0000	FED1	High
16	200	180	90	100	684	0.9999	FED2	Low
17	200	180	70	100	684	1.0000	FED3	Normal
18	200	180	60	100	684	0.9998	FED4	Normal
19	200	180	150	100	684	0.9999	FED5	High
20	200	180	130	120	684	0.9997	FEE1	High
21	200	180	130	110	684	0.9998	FEE2	High
22	200	180	130	90	684	0.9998	FEE3	High
23	200	180	130	80	684	0.9999	FEE4	High

Compared with research results from other studies (we used the concentration of the standard curve of each study to scan the absorbance spectrum), the results were stable under the same conditions of buffer (FLB) and chromogenic agent (FLC). In addition, the maximum absorption wavelength was not changed with the increase in the iron content (Fig.1).

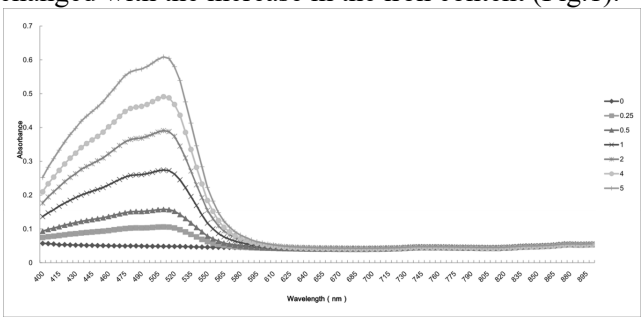

Fig.1 Absorption spectroscopy

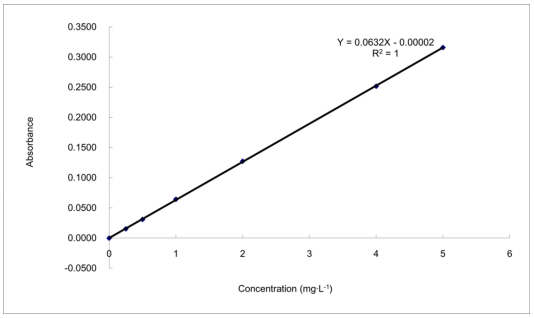

Fig.2 Iron calibration curve

3.2. The selection of the best experimental conditions

To obtain accurate results, and according to the principle of the experiment, we inspected the factors that may affect the chromogenic result for Fe^{2+} as follows: the read time, sample quantity, and dosage of FLA, FLB and chromogenic reagent FLC. According to the principle of the method, the tested values of all of the parameters are shown in Tab.1.

The iron determination method using the SmartChem200 automatic chemical analysis was changed on the basis of the national standard iron colorimetric method. For the adapted iron method and the dosage of reagents FLA, FLB and FLC, see test NO.1 that is listed in Tab.1. First, to search for the best read time on the basis of the parameters listed in test NO.1 in Tab.1, the modified parameters are presented in test NO.1, 2, 3, 4, 5 and 6. Next, after further consideration of the balance between absorbency, the correlation coefficient and the actual change of the absorbency of the fixed value of 2 $mg \cdot L^{-1}$ iron concentration, we determined the best read time as 684 s. Then, under this condition of a fixed read time of 684 s, and in view of the sample amount (test NO.1, 7, 8, 9, 10 and 11 in Tab.1), the dosage of the FLA reagent (test NO.1, 12, 13 and 14 in Tab.1), the dosage of the FLB reagent (test NO.1, 15, 16 17, and 18 in Tab.1) and the dosage of the chromogenic FLC reagent (test NO.1, 20, 21 22, and 23 in Tab.1), we determined the best iron method (test NO.17, the method name is FED3). This method was determined using the SmartChem200 automatic chemical analysis after much consideration of the balance between absorbency, the correlation coefficient and the actual change of the absorbency of the fixed 2 $mg \cdot L^{-1}$ iron concentration. Every parameter setting of the FED3 iron method is listed in Tab.2 (the absorbance value is not listed below). To verify the accuracy and stability of the measured data, we can insert a known concentration of standard sample between a certain number of samples during the process of determining the iron content of the plants.

Tab.2 Measurement condition settings

Sample and Reagents	Addition/μL	Delay or Read time/s	Other conditions
Sample	200		Wave length: 510 nm

Reagent 1 (FLA)	180	Delay 36 s	Measure method: Endpoint
Reagent 2 (FLB)	70	Delay 36 s	Linear: line
Reagent 3 (FLC)	100	Read 684 s	Reagent blank: use

3.3. The preparation of the iron standard curve

To prepare the 5 $mg \cdot L^{-1}$ iron standard solution, we used 0.5 ml 1000 $mg \cdot L^{-1}$ iron standard solution and added distilled water to dilute to a constant volume of 100 ml.

According to the experimental methods, the automatic chemical analyzer, which can mix a series of standard concentrations of 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 5.0 $mg \cdot L^{-1}$, was used with the best iron method (FED3) to determine the absorbance values for various points in the iron standard curve. The absorbance had a good linear relationship with the Fe^{2+} content in the range of 0.01 $mg \cdot L^{-1}$ to 5 $mg \cdot L^{-1}$ (data not listed, please see supplemental data in S1 Table.); the linear regression equation of the standard curve is Y=0.0632**X*-0.00002, and the correlation coefficient is 1.0000 (Fig.2). The iron content of Chinese fir leaves and roots is mostly between 0 and 5 $mg \cdot L^{-1}$ (data not listed, please see supplemental data in S2 Table.).

Compared with the conventional methods, the automatic chemical analyzer only required the highest standard solution (5.0 $mg \cdot L^{-1}$), and the standard curve of every other concentration can be automatically prepared by SmartChem200. This greatly reduced the error that can occur with the artificial addition of samples, avoided the tedious preparation of the standard curve, reduced the use of consumables in the experiment and reduced the experimental cost.

3.4. Accuracy and precision

The approaching degree of the measured value and actual value is called the accuracy. The difference between the measured value and the actual value is called the error. A high or low accuracy is always expressed in the error, i.e., the smaller the error the higher the accuracy of the analysis results. Precision is the degree that each measured value conforms to another and is determined by measuring the same sample several times with an emphasis on repeatability and reproducibility.

According to the identified FED3 method of determining iron content, 30 iron samples were analyzed with a concentration of 2.0 $mg \cdot L^{-1}$, which was prepared by the standard iron solution. The mean value was 2.0004, the standard error was 0.00625, and the standard deviation and variance were 0.03588 and 0.001, respectively (data not listed, please see supplemental data in S3 Table.).

Using this method to determine 5 samples, each sample was placed in 8 sample cups and the analysis was repeated 8 times; the mean, standard deviation, standard error and variance of each sample are shown in Tab.3.

Tab.3 The descriptive statistics of the samples

Sample	Mean	Standard error	Standard deviation	Variance
1[a]	1.2494	0.01539	0.4354	0.002
2[b]	0.9192	0.01757	0.04970	0.002
3[c]	0.8915	0.01454	0.04113	0.002
4[d]	0.3252	0.02393	0.06768	0.005
5[e]	0.3447	0.02430	0.06873	0.005
6[f]	0.1594	0.03556	0.07952	0.006

Note: a, b and c are the root samples of Chinese fir; d and e are the leaf samples of Chinese fir; f is a blank sample

3.5. The recovery rate of the standard addition

Using the FED3 method to determine the blank sample with a known iron concentration and an added fixed iron standard solution with a concentration of 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$, the analysis was repeated 3 times, and then the recovery rate of the blank standard addition was calculated based on the standard addition recovery calculation formula (Tab.4). Tab.4 illustrates that the recovery rate for the blank sample is between 91.05% and 113.24%, and the average recovery rate of the blank sample is 98.89%;

the results show that using this method can result in a better recovery rate. This further confirmed the accuracy of the instrument and the analysis method.

Tab.4 The recovery rate for the blank samples

Sample	Iron content/ （$mg \cdot L^{-1}$）	Addition concentration/ （$mg \cdot L^{-1}$）	Iron content after addition/（$mg \cdot L^{-1}$）	Recovery rate /(%)
1	0.1594	0.5	0.7256	113.24
2	0.1594	1.0	1.0699	91.05
3	0.1594	2.0	2.0072	92.39

Using the FED3 method, the analysis of a sample with unknown iron concentration and the addition of 0.05 ml of a fixed iron standard solution with a concentration of 50, 100 and 200 $mg \cdot L^{-1}$ to 4.95 ml was repeated 3 times, and then the average value and the recovery rate for standard sample addition was calculated based on the standard addition recovery calculation formula (Tab.5). Tab.5 shows that the recovery rate for the sample is between 91.05% and 114.72%, and the average recovery rate for the sample is 104.62%. This further demonstrates that this method can be used to determine the iron content of plants.

Tab.5 The recovery rate for the samples

Sample	Iron content/ （$mg \cdot L^{-1}$）	Addition concentration / （$mg \cdot L^{-1}$）	Iron content after addition /（$mg \cdot L^{-1}$）	Recovery rate /(%)
1-1[a]	1.2494	0.5000	1.8117	112.46
1-2[b]	0.9192	0.5000	1.4928	114.72
1-3[c]	0.3252	0.5000	0.8286	100.68
1-4[d]	0.3447	0.5000	0.8443	99.92
1-5[e]	0.1594	0.5000	0.7256	113.24
2-1[a]	1.2494	1.0000	2.3342	108.48
2-2[b]	0.9192	1.0000	2.0409	112.17
2-3[c]	0.3252	1.0000	1.3627	103.75
2-4[d]	0.3447	1.0000	1.3795	103.48
2-5[e]	0.1594	1.0000	1.0699	91.05
3-1[a]	1.2494	2.0000	3.302	102.63
3-2[b]	0.9192	2.0000	2.933	100.69
3-3[c]	0.3252	2.0000	2.4236	104.92
3-4[d]	0.3447	2.0000	2.5198	108.755
3-5[e]	0.1594	2.0000	2.0072	92.39

Note: a and b are the root samples of Chinese fir; c and d are the leaf samples of Chinese fir; e is the blank sample

4. Conclusion

The analysis results show that the method described in this article is simple, practical, accurate and reliable, with high reproducibility, and it can satisfy the requirement of determining the iron content of Chinese fir samples. Using the method in this article, Fe^{3+} was reduced to Fe^{2+} by hydroxylamine hydrochloride, and Fe^{2+} and 1,10-phenanthrolinemonohydrate generated an orange complex. This orange complex is stable over a certain period of time, but the time for measurement is limited, and a large number of samples cannot be determined quickly using the conventional method for determining iron content (i.e., the chromogenic reaction can be determined in 1.5 hours). In addition, the interfering factors, such as human error, are much greater in the traditional method.

Compared with the national standard method for determining iron content, using the SmartChem200 automatic chemical analyzer to determine the iron content of Chinese fir samples

improved the measurement precision and accuracy (with a high degree of automation), and the method of iron content determination that is described in this article has a fast analysis speed, less reagent consumption, little environmental pollution, and can be simply operated. Therefore, this method for determining iron content is particularly suitable for measuring large numbers of plant samples. In a word, we conclude that the method of determining iron content which described in this paper is a rapid, reliable, practical, and inexpensive method for determining iron content of plant tissues.

Supporting Information

S1 Tab. The absorbance of iron calibration curve.

S2 Tab. The iron content of Chinese fir leaves and roots.

S3 Tab. Using FED3 method of determining iron content of 30 samples with iron standard concentration of 2.0 $mg \cdot L^{-1}$.

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