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# Purity determination and uncertainty evaluation of folic acid by mass balance method

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ABSTRACT

Folic acid is one of the most important nutrient substances for human beings, especially for the pregnant women and infants. Therefore the purity determination of folic acid is particularly important. The mass balance method was employed to determine the purity of folic acid, by using the measures of high performance liquid chromatography (HPLC), Karl Fischer titration and other conventional approach. The moisture quantification of folic acid was a major problem since it is a thermally unstable substance and it is apt to contain crystal water. Therefore, a novel improved Karl Fischer method was established for accurate determination of the water content in folic acid, whose repeatability (RSD=2.9%) was significantly better than that of the original direct injection method (RSD=12%). The purity of folic acid certified reference material (CRM) determined by mass balance method was 90.9% with an expanded uncertainty of 0.35%, and the content of water (the major impurity) was 8.5%, with an expanded uncertainty of 0.32%.



## 1. Introduction

Folic acid (FA) otherwise known as vitamin M or pteroylglutamicacid [1], is the folate vitamin that is classed as water-soluble vitamins belonging to the B group [2]. It plays an important role in human nutrition [3], and is required for several fundamental biological processes, including nucleotide biosynthesis and amino acid metabolism, and is an essential component of the diet. Inadequate FA status is associated with several pathological conditions, including neural tube defects [4], anemia, cancer, Alzheimer disease [5] and congenital heart defects. Mandatory supplementation of foods with FA has been introduced in the United States and Canada and is being considered in China, Australia, Ireland, and the United Kingdom. Because FA is essential to human nutrition, it is important that it is accurately and rapidly quantified in food and medical samples [6].

Because of these concerns, analytical methods that accurately and precisely measure FA are needed to provide quality assurance and quality control for commercial products [7]. Several methods of folate analysis in foods are currently in use. The most common method is the microbiological assay, which relies on the folate-dependent growth of Lactobacillus rhamnosus measured by turbidity. This approach is slow and unsuitable for automated analysis [8,9]. A second approach is the measurement of folate by chemical means; both high-performance liquid chromatography (HPLC) and mass spectrometry (MS) methods have been developed [10–14]. Several chromatographic methods have been reported for the separation and quantification of FA and folate derivatives in foods [15–18], such as vegetables, flour, bread [19], beverages [20], seaweeds [21], vitamin-fortified fruit drinks [22], legumes, processed meats [23] and fortified wheat flour [24].

However, there has not been reported for the purity determination method of FA. An accurate and precise purity determination method is the basis of developing certified reference materials (CRMs), which are vital in chemical metrology. They are important in the development and validation of new methods, in the identification of biases among different methods, and ultimately, in the defense of claims of measurement traceability [25]. In the reference standard calibration process, chromatographic, spectrophotometric, phase solubility analysis or differential scanning calorimetry (DSC) are the usual methods used to determine the purity. The analytical methods to be employed in examining a substance should be considered in relation to its intended use [26]. For the characteristics of FA, the mass balance method is employed, which is to quantify all of the impurities (as well as moisture, volatile and ash) and subtract the sum of these impurities from 100%, without recourse to adding a reference material. In the mass balance method, the quality of the analytical data on impurities gathered by the most commonly used techniques, high-performance liquid chromatography (HPLC) or gas

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chromatography (GC), is more often dependent on the physical properties of a pure substance than on its structural complexity and can suffer when there are difficulties with sample preparation and the suitability and standardization of the detector [27]. Thermogravimetric analysis (TGA) may be used to determine the water and volatile content. Alternatively, the water content may be determined by Karl Fischer titration and the content of volatile solvents by GC. TGA or a muffle furnace method was used for ash analysis.

The barrier of the mass balance method for FA purity determination is moisture quantification. FA is thermally unstable, water-soluble substance that is apt to contain crystal water. Normally, the water content may be determined by Karl Fischer titration or TGA. Karl Fischer titration is based on a selective chemical reaction: the oxidation of sulfur dioxide by iodine in the presence of water with methanol as working medium [28]. It is more suitable for FA than TGA, which is a technique that measures changes in the mass of a sample as a function of temperature and time [29]. In recent studies, Karl Fischer titration was applied to determine the water content of pyrolysis oil [30], edible oils [31], honey [28], pollen [32] and so on [33]. But there has not existed an applicative moisture determination method for FA. In this assay, a novel Karl Fischer titration method was developed to make the moisture determination of FA to be reliable, accurate and precise. Based on this method, an accurate and precise purity determination method of FA by mass balance method was developed.

## 2. Experimental

### 2.1. Instruments

HPLC measurements were made on an Agilent 1200 with the diode-array detector (DAD) (Agilent Technologies, Palo Alto, USA). The moisture of a substance was determined by the Mettler-Toledo DL 39 Karl Fischer coulometric titrator (Greifensee, Switzerland). The present study also employed a Perkin-Elmer TGA-1 thermogravimetric analysis (Norwalk, CT, USA), a Sartorius balance (Inc., Cherry Hill, NJ, USA) and Agilent 6890N gas chromatograph (GC) measurements. Elemental Analyzer (CHNS) was Elementar Analysensysteme GmbH (Hanau, Germary) Vario EL cube with Thermal Conductivity Detector (TCD).

### 2.2. Reagents and materials

FA was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methanol of HPLC grade was purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate ($KH_2PO_4$) was obtained from a Johnson Matthey Company (Alfa Aesar, Shore Road, Heysham, Lancs, Ward Hill, MA, 98% purity). Ammonium hydroxide (for analysis, 28–30 wt% solution of $NH_3$ in water) was purchased from Acros (Geel, Belgium). It was diluted to 0.05% in water when using in experiments. Karl Fischer reagent (Coulomat AK) was purchased from Fluka (Buchs, Switzerland). Other reagents were analytical grade. Experimental water was all deionized water. Parafilm that was used as membrane in Karl Fischer titration method was purchased from Pechiney Plastic Packaging, USA.

### 2.3. Method

#### 2.3.1. Organic purity determination by HPLC

*2.3.1.1. Method of HPLC.* The identified organic purity was quantified by HPLC with DAD detector. Before the measurements, HPLC was calibrated by the solution of the measured compound. HPLC was equipped with an Inertsil ODS-C18 (250 mm × 4.6 mm × 5 μm) column (GL Science Inc. Japanese) and DAD detector at 284 nm. Sample (1 mg $mL^{-1}$) was prepared by dissolving 10 mg in 10 ml of 0.05% (v/v) ammonium hydroxide. The injection volume was 2 μL. The mobile phase was an isocratic elution of potassium dihydrogen phosphate/water (50 mmol) and methanol (85:15 v/v) with a flow rate of 1.0 mL $min^{-1}$. The solutions were degassed ultrasonically, freezed until required.

*2.3.1.2. Determination method of HPLC.* The organic purity ($P_o$) determined by HPLC can be calculated as follows:

$$P_o = \frac{f_0 A_0}{f_0 A_0 + \sum_{i=1}^{n} f_i A_i} \quad (1)$$

where $A_0$ and $f_0$ are the peak area and response factor of FA, respectively; $A_i$ and $f_i$ are the peak area and response factor of impurity $i$ ($i=1\sim n$), respectively. The impurities that did not absorb at this wavelength (or else UV range) are not included here.

*2.3.1.3. Uncertainty method of HPLC.* The uncertainty method of HPLC ($u(P_o)$) can be calculated as follows:

$$u_{rel}(P_o) = \sqrt{u_{rel,1}^2 + u_{rel,2}^2 + u_{rel,3}^2 + u_{rel,4}^2} \quad (2)$$

where $u_{rel,1}$ is the uncertainty from the relative standard deviation (RSD) of HPLC; $u_{rel,2}$ is the uncertainty from difference of response factors of impurities of HPLC, it can be calculated as follows:

$$u_{rel,2} = \frac{1}{\sum B_i}\left(\frac{\sqrt{\Sigma u_{2-i}^2}}{\sqrt{3}}\right) \quad (3)$$

$$u_{2-i} = B_{i\ \max\lambda} - B_{i\ \text{value}\ \lambda} = \frac{A_{i\max\lambda}}{\Sigma A_{i\ \text{value}\ \lambda}} - \frac{A_{i\ \text{value}\ \lambda}}{\Sigma A_{i\ \text{value}\ \lambda}} \quad (4)$$

where $A_{i\ \text{value}\lambda}$ is the peak area in the determined wavelength; $A_{i\ \max\lambda}$ is the peak area in the maximum wavelength especially for the impurity $i$. $\Sigma B_i$ is the sum of area for all components, which is always equal to 1.

The $u_{rel,3}$ is the uncertainty of liner detection, which is determined by instruments; the $u_{\ rel,4}$ is the uncertainty of limit of detection (LOD). And it can be calculated as follows:

$$u_{rel,4} = C_{LOD}/C_{value} \quad (5)$$

$$C_{LOD} = 2NC/H \quad (6)$$

where $N$ means noise height, it was 0.02 in this assay; $C_{LOD}$ means the lowest concentration that can be detected; $H$ means the peak height of the lowest point, which can be detected; $C$ means the concentration of lowest point that can be detected. $C_{value}$ means the concentration of FA sample during quantification.

#### 2.3.2. Moisture method

*2.3.2.1. Methods of moisture determination*

*2.3.2.1.1. Moisture method of TGA.* The instrument was kept at 50 °C and purged with a constant flow of nitrogen; measurements were performed under helium atmosphere. Approximately 6–7 mg portions of the samples were accurately weighed in open standard aluminum crucibles. Thermograms were determined by heating the samples from 30 °C up to 200 °C with a heating rate of 10 °C $min^{-1}$, followed by an isothermal step for 300 s.

*2.3.2.1.2. Moisture method of direct sampling Karl Fischer titration.* Titration conditions were a sample mixing time of 30 s, titration end point selection of automatic termination, the magnetic stirring rod rotate speed of 40r $s^{-1}$, a start and stop drift of 2 μL $min^{-1}$, a polarization current of 2 μA and an endpoint detection voltage of 100 mV. Sample masses of 3 mg were accurately weighed.

In the direct sampling, after weighing, the cover was opened for 10 s to inject sample directly without any other means to protect from the air. For the blank experiments, the cover was opened for 10 s without injection, and the same condition and operation as that of sample was applied. All results of the automated determinations were calculated referring to the obtained water contents by manually conducted Karl Fischer titration and are mostly displayed as recovery rate in μg of the absolute weight of water. Measurements were taken at respective room temperatures and humidity.

*2.3.2.1.3. Moisture method of copper-pan sampling Karl Fischer titration.* The technical parameters of Karl Fischer titration was the same as direct sampling, but the operating steps were as follows a copper-pan with stick was made to inject the sample of the Karl Fischer titration. Firstly, the sample was accurately weighed in the copper-pan, and then the stick penetrated though the gasket of the cover, at last the sealing membrane was wrapped around the copper-pan to protect from air. The cover with the copper-pan was put in the Karl Fischer titration instrument, as shown in Fig. 1 (left). Secondly, the water within the titration cell when opened cover of injecting was titrated by Karl Fischer titrator and an equilibrium drift was achieved. Thirdly, pushing the stick made the copper-pan into the titrant, and the sample would be dispersed by the magnetic stirring rod, as shown in Fig. 1 (right).

*2.3.2.1.4. Moisture method of glass-pan sampling Karl Fischer titration.* The technical parameters of Karl Fischer titration was the same as direct sampling, but the operation was as follows; firstly, the sample was accurately weighed in the glass-pan, and then the glass-pan was fixed with the cover by sealing membrane in order to protect the sample from the air, as shown in Fig. 2 (left). Secondly, the water within the titration cell when opened cover of injecting was titrated by Karl Fischer titrator and an equilibrium drift was achieved. Thirdly, the glass-pan was pushed into the titrant by an iron needle penetrating through the gasket of the cover, and the sample would be dispersed by the magnetic stirring rod, as shown in Fig. 2 (right). Pay attention that the iron needle would not meet the titrating solution anymore.

*2.3.2.1.5. Method of elemental analysis.* The technical parameters: the temperature of oxidation tube was 1150 °C; the time of oxygen atmosphere was 90 s; the speed of oxygen was 40 mL min$^{-1}$; the temperature of reduction tube was 850 °C and the speed of helium was 230 mL min$^{-1}$.

*2.3.2.2. The quantification method of Karl Fischer titration.* The moisture content ($X_w$) determined by Karl Fischer titration can be calculated as follows:

$$X_w = \frac{m'}{m}\frac{1}{a_1 a_2 a_3} \tag{7}$$

where $m$ is the weight of sample (FA); $a_1$ is electrolytic efficiency; $a_2$ is the ratio of iodine and water; $a_3$ is the water mass released and $m'$ can be calculated as follows:

$$m' = \frac{M}{n}\frac{it}{96485} \tag{8}$$

where $M$ is the relative molecular weight of water (18.0 g mol$^{-1}$); $i$ is electrolytic current; $t$ is the time of electrolysis; $n$ is the number of electron in Karl Fischer reaction ($n=2$).

*2.3.2.3. Uncertainty method of Karl Fischer titration.* In the case of a water determination by Karl Fischer titration, the combined uncertainty $u(X_w)$ can be calculated by

$$u(X_w) = X_w\sqrt{u_A{}^2 + \left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(m')}{m'}\right)^2 + \left(\frac{u(a_1)}{a_1}\right)^2 + \left(\frac{u(a_2)}{a_2}\right)^2 + \left(\frac{u(a_3)}{a_3}\right)^2} \tag{9}$$

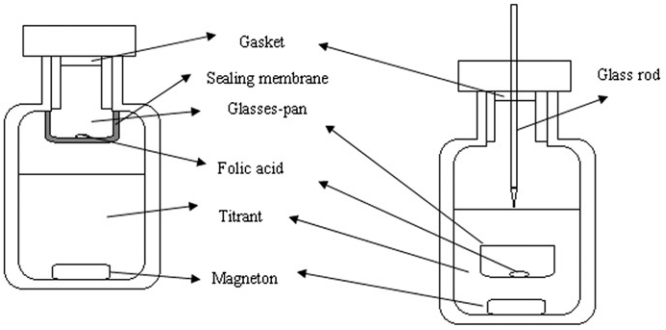


**Fig. 2.** The injection process of glass-pan Karl Fischer titration method.

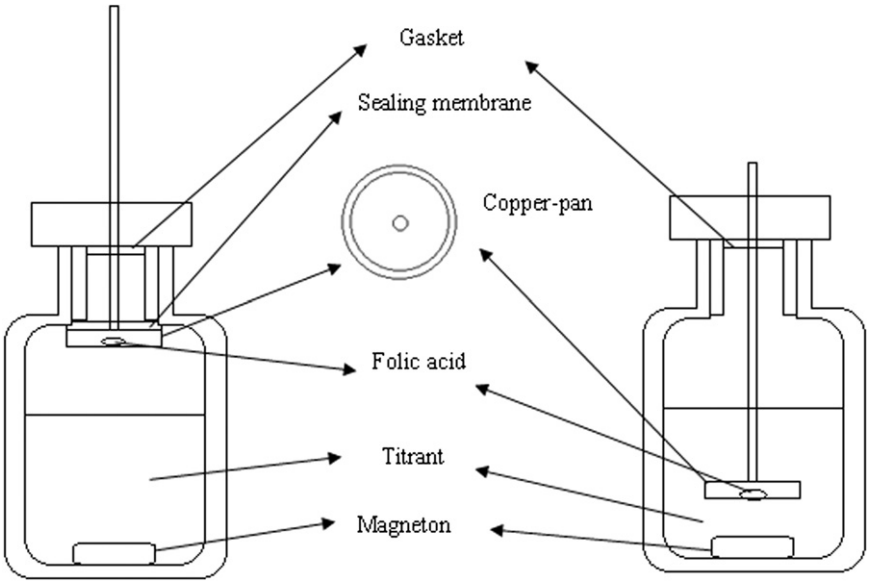


**Fig. 1.** The injection process of copper-pan Karl Fischer titration method.

$u_A$ is RSD of Karl Fischer titration; $u(m)$ is determined by the uncertainty of the balance; $u(m')$ is determined by the uncertainty of limit of detection (LOD) and $u(a_1)/a_1$ is estimated to 0.50%, which assumption is electrolytic efficiency of generated iodine near 100%. In theory, the ratio of iodine and water is 1:1, but there are side effects in fact. The deviation of value and true value is about 1.3% [25], which is the value of $u(a_2)/a_2$ here. Because FA is solid sample, $u(a_3)/a_3$ can be estimated to 2% for the water which is not released.

#### 2.3.3. Determination of ash content by TGA method

*2.3.3.1. Method of TGA.* Before the TGA experiment, the empty crucible was burned to be clean and then weighted by balance, which was $W_0$. The instrument was kept at 50 °C and purged with a constant flow of nitrogen; measurements were performed under helium atmosphere. Approximately 10 mg portions of the samples were accurately weighed in open standard aluminum crucibles. Thermograms were determined by heating the samples from 50 °C up to 850 °C with a heating rate of 10 °C $min^{-1}$, followed by an isothermal step for 20 mins. The weight of crucible with sample was weighted before and after burning was $W_1$ and $W_2$ respectively.

*2.3.3.2. Determination method of TGA.* The ash content ($X_a$) determined by TGA can be calculated as follows:

$$X_a = \frac{W_2 - W_0}{W_1 - W_0} \tag{10}$$

where $W_0$ is the weight of empty crucible; $W_1$ and $W_2$ is the total weight before and after burning respectively.

*2.3.3.3. Uncertainty method of TGA.* In the case of ash, the combined uncertainty $u(X_a)$ can be calculated from:

$$u(X_a) = X_a \sqrt{u_A{}^2 + \frac{u(W_2)^2 + u(W_1)^2}{(W_2 - W_0)^2} + \frac{u(W_1)^2 + u(W_0)^2}{(W_1 - W_0)^2}} \tag{11}$$

where $u_A$ is the uncertainty from the RSD of ash determined; $(W_1 - W_0)$ means the masses of sample and $(W_2 - W_0)$ means the ash.

#### 2.3.4. Determination of volatile impurities by GC method

Two different solvent (methanol and acetonitrile) was used to dissolve the FA sample. Volatile impurities were ignored when no GC peak was observed that could not be attributed either to the solvent or to FA. The determination ($X_v$) and uncertainty $u(X_v)$ method could be left out.

#### 2.3.5. Mass balance calculation

The mass balance method involves quantifying all of the impurities, including moisture, ash and volatile, and subtracting the sum of these impurities from 100%, according to which the content of the analyte can be calculated as follows [27]:

$$\text{Content\%} = \text{organic purity\%}(1 - \text{water\%} - \text{volatile\%} - \text{ash\%})100\% \tag{12}$$

Organic purity analysis of the reference standard candidates was carried out by HPLC methods. Water content of the reference standard candidates was determined by the improved Karl Fischer titration method (glass-pan Karl Fischer titration method). Volatile in the reference standard candidates were determined by the GC method. Ash determinations were carried out by routine method of TGA.

In this paper the expression above would be written in short as follows:

$$P = P_o(1 - X_v - X_w - X_a) \tag{13}$$

where $P$ is the content of FA; $P_o$ is the organic purity determined by HPLC; $X_v$ is the volatile impurities content determined by GC; $X_w$ is the moisture content determined by glass-pan Karl Fischer titration, and $X_a$ is the ash content determined by TGA. If the content of volatile, ash or water is below 0.1%, it could be ignored in the results of FA purity determination and the uncertainty calculated.

#### 2.3.6. Measurement uncertainty in the mass balance method

In the case of assay, the combined uncertainty $u(P)$ can be calculated as follows:

$$u(P) = P\sqrt{\left(\frac{u(P_o)}{P_o}\right)^2 + \frac{u^2(X_v) + u^2(X_w) + u^2(X_a)}{(1 - X_v - X_w - X_a)^2}} \tag{14}$$

The calculated method of each part ($P_O$, $u(P_o)$, $u(X_w)$, $u(X_v)$, $u(X_a)$) of this formula has been shown above.

## 3. Results and discussion

### 3.1. Optimization of HPLC conditions

In their study, Ruggeri et al. [23] used a mobile phase containing acetonitrile and phosphate buffer solution to determine the content of FA in an Italian reference diet by HPLC method. Alternatively, Cheung et al. [2] used methanol instead of acetonitrile. In this assay, when using acetonitrile, FA cannot be well separated from the impurities under the existing conditions, so methanol was chosen. The percentages of organic (methanol) and aqueous (phosphate buffer) mobile phase components ranged from70% to 10% and from 30% to 90% (v/v), respectively. Isocratic elution starting with 15% methanol and 85% phosphate buffer solution (v/v) at a flow rate of 1.0 mL $min^{-1}$ was the best proportion to obtain the optimal peak sharpness and efficient separation from impurities. Each mobile phase composition results were summarized in Table 1. After establishing the chromatography conditions, the best analytical UV-wavelength to detect FA was 284 nm (Fig. 3, Table 2).

### 3.2. Purity determination by HPLC

Fig. 4 shows the typical chromatogram of FA. The main peak at 25.834 min indicates FA, and the peaks at 3.657, 4.408, 5.436, 8.644, 12.947 and 21.681 min were the impurities. The purity of analyte was determined by an area normalization method.

After calculating by Eq. (1) and Table 3, the mean of purity is 99.330%. As the organic impurities are below 1%, so the impurity and the main ingredients are supposed to be the similar

**Table 1**
Each mobile phase composition results.

| Mobile phase (A): aqueous (phosphate buffer ~0.05 mmol/ml, pH 5.36) and (B):organic (methanol). | |
|---|---|
| Mobile phase components | Results |
| A:B=30:70 | 2 min peak; not separated |
| A:B=70:30 | 4 min peak; not separated |
| A:B=80:20 | 15 min peak; impurities not separated |
| A:B=85:15 | 25 min peak; better separation |
| A:B=90:10 | 50 min peak; better separation, but time is too long |

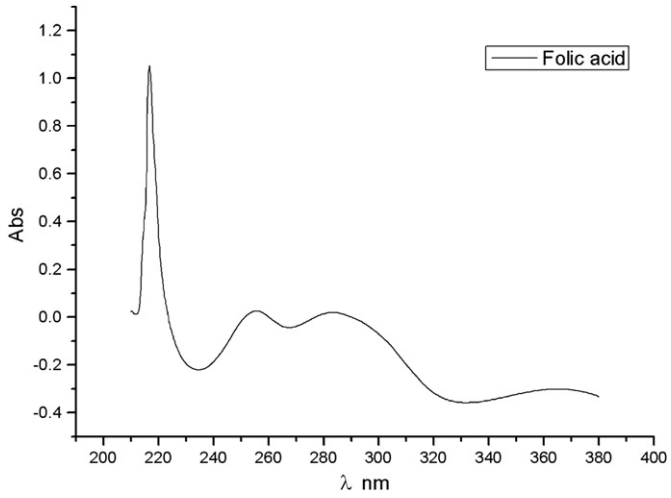


**Fig. 3.** Folic acid UV wavelength scan (200–400 nm).

**Table 2**
Folic acid wavelength scan results (UV 200–400 nm).

| Peak no. | Start (nm) | Apex (nm) | End (nm) | Height (Abs) | Valley (nm) |
|---|---|---|---|---|---|
| 1 | 380.0 | 365.7 | 331.4 | 0.1784 | 331.4 |
| 2 | 331.4 | 283.3 | 268.0 | 0.4985 | 268.0 |
| 3 | 268.0 | 256.0 | 234.6 | 0.5059 | 234.6 |
| 4 | 234.6 | 216.7 | 210.0 | 1.5314 | 210.0 |

(Apex: it was the height of the top absorbed point in UV.)

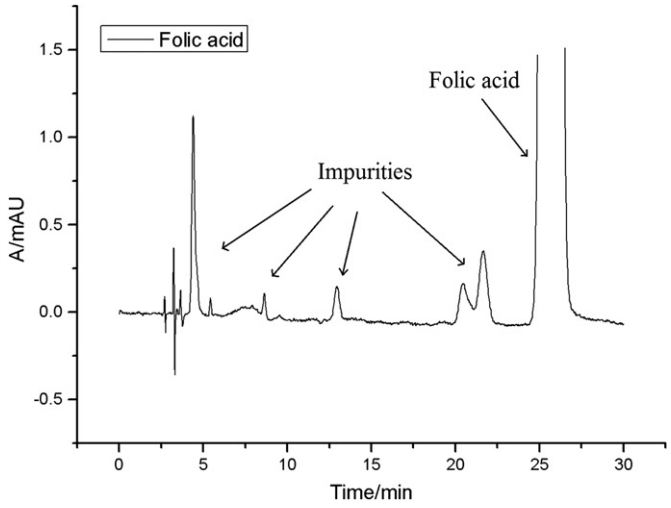


**Fig. 4.** The typical chromatography of folic acid.

**Table 3**
Purity of analyte from HPLC methods.

| Measurement | HPLC (%) |
|---|---|
| Purity | 99.311 |
| | 99.390 |
| | 99.368 |
| | 99.329 |
| | 99.321 |
| | 99.318 |
| | 99.304 |
| | 99.308 |
| | 99.322 |
| $\overline{X}$ | 99.330 |
| RSD | 0.029 |

compounds, so differences in their response factors are small. Therefore, the difference in response factor for major component and impurities upon selected wavelength is ignored in value assignment, but is calculated in evaluation of uncertainty. Other experimental conditions were the same as the above.

### 3.3. Uncertainty of HPLC

The $u_{rel,1,}$ was estimated as 0.029% ($n=9$) as shown in Table 3. The $u_{rel,2}$ of HPLC method could also be assessed according to Eqs. (3) and (4). The result which had been calculated was 0.041% (Table 4). The related coefficient of the liner was 0.99999 after calculating from the data of Table 5, it could be included in the liner range of equipment. So $u_{rel,3}$ can be ignored. The $u_{rel,4}$ can be calculated by Eqs. (5) and (6) and the data of Table 5, the result of $u_{rel,4}$ was 0.000175% which can be ignored. The uncertainty of HPLC can be calculated by Eq. (2), then $u_{rel}(P_o)$ was 0.050%.

### 3.4. Optimization of moisture method and determination of water content

As the moisture method mentioned above, the TGA method was not suitable for the thermally unstable substance something like FA. When using TGA to determine the moisture of FA, it will be losing weight that cannot reach the stable state, as shown in Fig. 5; the curve was not stable at last. This experiment was also tried the heating rate of 1 °C $min^{-1}$, but the result was similar to Fig. 5. (the temperature of folic acid altered is about 130 °C.) So the data of this method was not reliable.

The second method is direct sampling Karl Fischer titration, this method was inflenced by the air moisture and the environmental factors, so the RSD (12%) of the result was too high to be accepted (Table 6).

Becasuse of the high RSD of the direct sampling Karl Fischer titration method and the high absolute value of blank water mass, the copper-pan method was used to make the results stable. But the titrant was reacted with the copper-pan, the copper was electrolysed and then separated out. It was harmful to the electrode, so this method could not be used anymore.

The last and the best way was glass-pan method, it could make the data stable and effectively protect the sample from the air moisture and the environmental factors. The air was well protected from the sample, and the blank that can be detected was about zero. The RSD (2.8%) was much lower than the driect sampling method, the data was as shown in Table 7. This method would be a useful novelty one for the users of KF, who were disturbed by the unstable environmental water or other factors. The method recommended here is considered an alternative technique whether the FA in any sample or the water content of it, also for these substances that has crystal water and thermally unstable like water-soluble vitamins or some natural products that have this features.

In addition, this method is a simple and effective way (no valve or tube) for any reaction which prevents any contact before the reaction and need fully contact during the reaction.

In summary, the glass-pan sampling method was the best way to determine the moisture of the FA. From Table 7 and Eqs. (7) and (8), the water content of FA($X_w$) was 8.49%.

The assumption of Elemental Analyzer is the sample contains only FA and hydrogen ($C_{19}H_{19}N_7O_6 \cdot xH_2O$), and then the moisture content can be calculated by percentage of H in the sample. The result of Elemental Analyzer (CHNS) which was calculated by hydrogen of the moisture was 8.56%. It was agreed with the glass-pan Karl Fischer titration method. But this method was not reliable for quantification in the final results because this research cannot meet its assumptions (actually, there are some organic

**Table 4**
The different wavelength of folic acid and the impurities in HPLC.

| Measurement | Retention time | Peak area in 220 nm (mAU min) | Peak area in 254 nm (mAU min) | Peak area in 270 nm (mAU min) | Peak area in 284 nm (value λ) (mAU min) | Peak area in 365 nm (mAU min) | Peak area in 380 nm (mAU min) | The impurity of $u_{2\text{-}i}$ | $u_{rel.2}$ |
|---|---|---|---|---|---|---|---|---|---|
| Impurity 1 | 3.657 | 1.00 | 0.49 | 0.74 | 0.80 | 0.39 | 0.00 | 0.003% | 0.041% |
| Impurity 2 | 4.408 | 11.60 | 8.50 | 17.30 | 15.30 | 0.00 | 0.00 | 0.027% | |
| Impurity 3 | 5.436 | 0.98 | 0.58 | 1.10 | 0.53 | 0.51 | 0.00 | 0.008% | |
| Impurity 4 | 8.644 | 1.70 | 1.00 | 1.80 | 1.20 | 0.82 | 5.90 | 0.064% | |
| Impurity 5 | 12.947 | 3.40 | 2.80 | 3.30 | 4.20 | 1.10 | 0.00 | 0.000% | |
| Impurity 6 | 21.681 | 24.80 | 14.40 | 21.80 | 23.80 | 7.00 | 5.40 | 0.014% | |
| Folic acid | 25.834 | 6177.80 | 2958.10 | 6347.90 | 7248.60 | 1607.00 | 1057.1 | 0.00% | |

**Table 5**
The data of folic acid LOD.

| Concentration of Folic acid (mg/ml) | Peak area | Peak height (mAU) |
|---|---|---|
| $6.113 \times 10^{-6}$ | 7.50 | 0.14 |
| $6.286 \times 10^{-5}$ | 10.50 | 0.19 |
| $9.193 \times 10^{-5}$ | 11.60 | 0.21 |
| 0.0009606 | 54.03 | 0.90 |
| 0.009779 | 496.37 | 8.87 |
| 0.09907 | 4296.33 | 74.03 |
| 1.016 | 45429.27 | 547.90 |

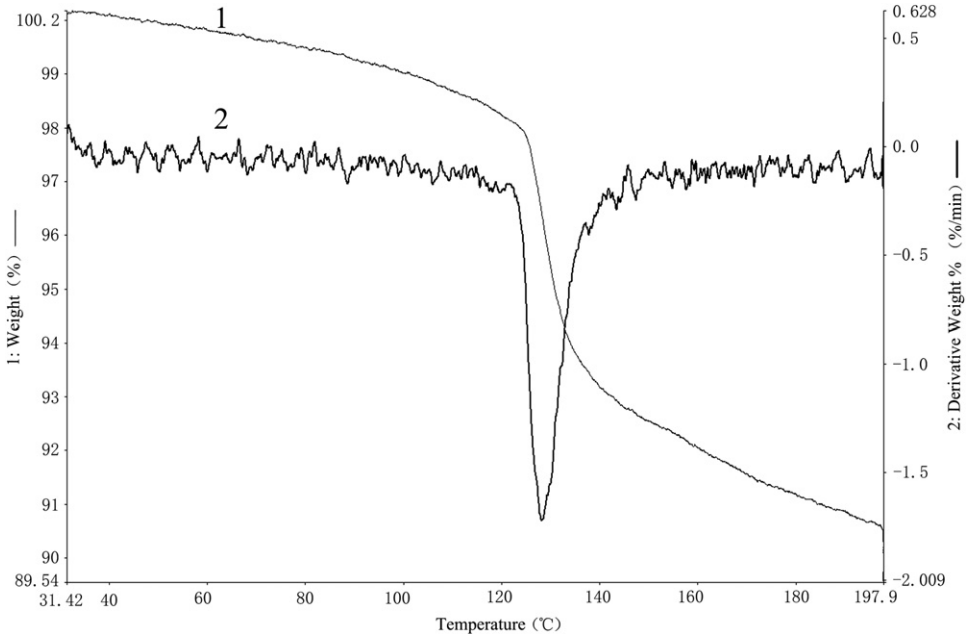


**Fig. 5.** The weight lost curve of TGA.

impurities in the sample); therefore, the result was used as a circumstantial evidence for the result of the Karl Fischer titration method. At last, another experiment of glass-pan Karl Fischer titration method was used to determine the moisture of the FA which had been vacuum dried for 3 days. The result was 7.69%, which was agreed with the moisture of FA theoretical (7.5%). The difference of the water content before and after vacuum drying was the surface absorbed water.

### 3.5. Uncertainty of water content

From glass-pan method, the RSD ($n=16$) was reduced from 12% to 2.8% (Tables 6 and 7).

From Eq. (9) and its explanation, the $u(X_w)$ was 0.32%.

### 3.6. Purity determination by the mass balance method

According to Eq. (13) and the results had been calculated as above. Also, the content of volatile and ash could be ignored in the results of FA purity determination, because they were below 0.1%. So Eq. (13) can be in short as:

$$P = P_o(1-X_W) \tag{15}$$

So the purity of FA was 90.9%.

### 3.7. Uncertainty of the mass balance method

As in this issue, the volatile impurities and ash was below 0.1%, they can be ignored. And the uncertainty of organic and moisture

**Table 6**
The data of the direct sampling KF titration.

| | Mass of Folic acid (μg) | Water mass (μg) | Blank water mass (μg) | Absolute water mass (water-blank mass average) (μg) | Moisture (%) |
|---|---|---|---|---|---|
| 1 | 2910 | 331.57 | 116.28 | 278.32 | 9.56 |
| 2 | 3090 | 337.82 | 21.17 | 284.57 | 9.21 |
| 3 | 2940 | 323.42 | 44.58 | 270.17 | 9.19 |
| 4 | 2990 | 254.72 | 41.33 | 201.47 | 6.74 |
| 5 | 2920 | 268.96 | 47.48 | 215.71 | 7.39 |
| 6 | 2940 | 226.99 | 67.20 | 173.74 | 5.91 |
| 7 | 3030 | 300.70 | 47.88 | 247.45 | 8.17 |
| 8 | 2920 | 280.05 | 40.65 | 226.80 | 7.77 |
| 9 | 3050 | 312.59 | 68.02 | 259.34 | 8.50 |
| 10 | 2920 | 291.86 | 49.84 | 238.61 | 8.17 |
| 11 | 2960 | 326.71 | 36.69 | 273.46 | 9.24 |
| 12 | 2920 | 267.40 | 57.93 | 214.15 | 7.33 |
| 13 | 2950 | 270.06 | | 216.81 | 7.35 |
| 14 | 2940 | 273.10 | | 219.85 | 7.48 |
| 15 | 3020 | 298.30 | | 245.05 | 8.11 |
| 16 | 3020 | 297.98 | | 244.73 | 8.10 |
| Average | | | 53.25 | | 8.01 |
| RSD | | | 44% | | 12 |

**Table 7**
The data of the galsses-pan sampling KF titration.

| | Weight of Folic acid (μg) | Water weight (μg) | Blank water weight (μg) | Absolute water weight (water-blank weight average) (μg) | Moisture (%) |
|---|---|---|---|---|---|
| 1 | 3010 | 254.06 | 0.00 | 254.06 | 8.44 |
| 2 | 3000 | 260.60 | 0.00 | 260.60 | 8.69 |
| 3 | 2960 | 262.69 | 0.00 | 262.69 | 8.87 |
| 4 | 3060 | 258.91 | 0.00 | 258.91 | 8.46 |
| 5 | 3070 | 248.18 | 0.00 | 248.18 | 8.08 |
| 6 | 3030 | 263.50 | | 263.50 | 8.70 |
| 7 | 3020 | 257.08 | | 257.08 | 8.51 |
| 8 | 3090 | 247.98 | | 247.98 | 8.03 |
| 9 | 2980 | 255.14 | | 255.14 | 8.56 |
| 10 | 3090 | 262.34 | | 262.34 | 8.49 |
| 11 | 3020 | 253.63 | | 253.63 | 8.40 |
| 12 | 3060 | 248.87 | | 248.87 | 8.13 |
| 13 | 3020 | 259.69 | | 259.69 | 8.60 |
| 14 | 2990 | 262.88 | | 262.88 | 8.79 |
| 15 | 3010 | 256.42 | | 256.42 | 8.52 |
| 16 | 2920 | 248.85 | | 248.85 | 8.52 |
| Average | | | 0.00 | | 8.49 |
| SD | | | | | 0.24 |
| RSD | | | | | 2.8 |

**Table 8**
The data of the mass balance method.

| | Organic (%) | Volatile | Moisture (%) | Ash | Mass balance (%) |
|---|---|---|---|---|---|
| Content | 99.33 | Below 0.1% | 8.5 | 0.03% | 90.9 |
| Uncertainty | 0.050 | Ignored | 0.32 | Ignored | 0.35 |

content had been calculated as above. The uncertainty can be calculated by Eq. (14), and the data of mass balance method is shown in Table 8.

So the purity of FA determined by mass balance method was 90.9% with an extended uncertainty of 0.35%.

### 3.8. Conclusion

In this study, a mass balance method was successfully developed for the purity determination of FA. The moisture quantification is a major problem in the mass balance method since FA is a thermally unstable substance and is apt to contain two molecules of crystal water. Therefore, an improved Karl Fischer titration method (glass-pan method) was established for accurate determination of the water content in FA. The purity of FA determined by mass balance method was 90.9% with an extended uncertainty of 0.35%. And the water content was 8.5%; the RSD was 2.8% by the new method. It was much better than the original method (RSD=12%). When the FA had been vacuum dried for 3 days, the moisture result was 7.69%, which was agreed with the moisture of FA theoretical (7.5%). This proved that the FA had two molecules of crystal water. The difference of the water content before and after vacuum drying must be the surface absorbed water. The method recommended here is considered an alternative technique whether the FA in any sample or the water content of it, especially for these substances that has crystal water and thermally unstable. This method was also suitable for the unstable water-soluble vitamins like vitamin C or some natural products which are unstable and have much water content like ginsenosides.

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