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Purity assignment of 17β-estradiol by mass balance method

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Abstract: In general, quantitative chemical analysis in various areas including food, the environment, in vitro diagnostics, etc., requires traceability in order to increase the reliability of the measurements. Measurement traceability is a property of an unbroken chain of comparisons relating an instrument's measurements to SI units. Purity analysis is the first process for establishing traceability to SI units in chemical measurements. The purpose of this study is to develop and validate a method of purity assignment for establishing the traceability of 17β-estradiol measurements in an in vitro diagnostics field. The establishment of this method is very important as it can be applied to the development of CRM and to the analysis of the purity of other hormones. The method of assignment of the purity of 17β-estradiol was developed using the mass balance method and was validated through participation in an International comparison. In the mass balance method, impurities are categorized into four classes as follows: total related structure impurities, water, residual organic solvents, and nonvolatiles/inorganics. In this study, total related structure impurities were characterized by a gas chromatography-flame ionization detector (GC-FID) and a high-performance liquid chromatography-ultraviolet (HPLC-UV) detector, water content was determined by a Karl-Fisher coulometer, and total residual solvents and nonvolatiles/inorganics were checked simultaneously by thermogravimetric analysis (TGA). The purity of the 17β-estradiol was 985.6 mg/g and the expanded uncertainty was 2.1 mg/g at 95% confidence. The developed method can be applied to the development of certified reference materials, which play a critical role in traceability.

Key words: 17β-estradiol, purity analysis, mass balance method, traceability, certified reference material

1. Introduction

Measurement is an assignment of a number to a characteristic of an object or event; this number can be compared with those of other objects or events. Quantitative measurement exists in various fields such as food, environment, pharmacy, and in vitro diagnostics. These days, routine quantitative analysis requires traceability to SI units for reliability and quality of measurement.[1] In the majority of cases, this traceability to SI units is ultimately achieved through linkage to a calibrator material of the defined species, of which the purity is established in a manner that is also traceable to SI units.[2-3] This

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has, in turn, led to the need to clearly determine the purity of a pure material. The aim of purity analysis is to provide traceability to the primary calibrators that are used in measurement. The ability to perform purity assignment of primary calibrators is regarded as a core technical competency for any institute wishing to claim metrological traceability for the results of measurement services.[4] Therefore, international comparisons for purity analysis have been organized and carried out by the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM).[5-9]

In general, purity is represented as the mass fraction of the main component present in a pure substance and can be expressed as Eq. (1).

$$P_A = \frac{m_A}{m_A + \sum m_x} \quad (1)$$

where, P_A = the mass fraction of the main component A in the pure substance, m_A = the mass of A in a gravimetrically defined aliquot of the material, and Σm_x = the combined mass of *1, 2, 3, ..., x* minor components (impurities) present in the pure substance. This method is referred to as mass balance method or the summation of impurities; impurities are determined by an independent method herein. In this paper, the mass balance method has been used to determine the purity of 17β-estradiol.

The purpose of this study is to develop and validate a method for the purity assignment of 17β-estradiol for the establishment of traceability of 17β-estradiol measurement at in vitro diagnostics field. The establishment of this method is of great significance in that it is applicable to the development of certified reference material (CRM) and to the purity analysis of other hormones. In the mass balance method, impurities that require assessment and quantification are categorized into four classes as follows: total related structure impurities, water, residual organic solvents, and nonvolatiles/inorganics.[5] Total related structure impurities were characterized by high performance liquid chromatography and gas liquid chromatography with a detector sensitive to common organic compounds, water content was determined by a Karl-Fisher coulometer, and nonvolatiles/inorganics and residual organic solvents were checked by thermo gravimetric analyzer (TGA). The developed method was validated through participation in international comparison organized by CCQM.

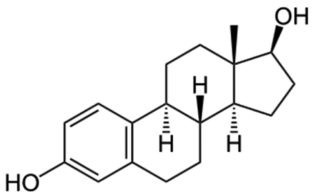

Fig. 1. Structure of 17β-estradiol.

17β-estradiol is a widely-used steroid pharmaceutical that is presented in biological matrices and is routinely tested for by clinical and diagnostic laboratories. Estradiol, or more precisely, 17β-estradiol, is a steroid and estrogen sex hormone, and the primary female sex hormone as shown in *Fig.* 1.[10-11] 17β-estradiol is important in the regulation of the estrous and menstrual female reproductive cycles. 17β-estradiol helps with the development and maintenance of female reproductive tissues such as uterus, fallopian tubes, and breasts, but it also essential for bone and joint health in females. In most laboratories, serum 17β-estradiol levels range from 20 to 80 pg/mL during the early to midfollicular phase of the menstrual cycle and peak at 200 to 500 pg/mL during the preovulatory surge. During the midluteal phase, serum 17β-estradiol levels range from 60 to 200 pg/mL.[12-14] While estrogen levels in men are lower than those in women, estrogens have essential functions in men as well.[15-16] A sample of 17β-estradiol was disseminated by the CCQM pilot lab. The purity of 17β-estradiol was calculated at (985.6 ± 2.1) mg/g within a 95 % confidence interval. The developed methods can also be applied to the development of certified reference materials and to the purity analysis of other hormones. Uncertainty budget was evaluated according to the international guide.[17-18]

2. Experimental

2.1. Chemicals

Commercial 17β-estradiol was purchased from

Sigma-Aldrich (≥ 99 %, USA). 17β-estradiol for CCQM key comparison was obtained from the CCQM pilot lab. Methanol was purchased from Burdick & Jackson (HPLC grade, USA) and filtered with a 0.2 μm PVDF filter (Supelco, USA) before use. Anhydrous ethanol was purchased from Sigma-Aldrich (≥ 99 %, USA). Water was purified through an osmosis filter system (Millipore Alpha Q, USA) and distilled with alkalic potassium permanganate. Distilled water was filtered and degassed with a 0.2 μm PVDF filter (Supelco, USA) before use.

2.2. Analysis of Impurities

2.2.1. Analysis of total related structure impurities

Total related structure organic impurities were characterized using GC-FID (Agilent technologies 6851N, USA) and HPLC-UV (Waters, USA) without any other derivatization process. The GC column was DB-5 (60 m × 0.53 mm, 0.25 μm film), and the oven temperature programming was as follows: 100 ºC (initial)-20 ºC/min-200 ºC (60 min)-5 ºC/min-250 ºC (90 min). Sample volume was 1 μL and was injected on column mode. 17β-estradiol was dissolved in ethanol; the concentration of the sample was about 1,000 mg/kg.

These impurities were also characterized using HPLC-UV (diode array detector). The HPLC column was Thermo BDS Hypersil C_{18} (250 × 4.6 mm, 5 μm); the mobile phase was used with gradients of water and methanol as follows: 50 % methanol (50 min)-90 % methanol (100 min) - 50 % methanol (10 min). The flow rate was 0.5 mL/min; the sample volume was 5 μL. The concentration of the sample was also about 1,000 mg/kg.

2.2.2. Determination of water content

Water content was determined using a Karl Fisher Coulometer (Metrohm, Switzerland). Because 17β-estradiol is soluble in methanol, which is a reagent solvent, water content measurement of 17β-estradiol was carried out by putting the sample directly into the reagent bottle. Because 17β-estradiol is very hygroscopic, it was immediately put into the reagent bottle after weighing. Sample was put into the vessel while the drift of the rate was in the range of 10-12 μg/min.

2.2.3. Analysis of nonvolatile/inorganic impurities

Nonvolatile/inorganic impurities were checked by TGA (Mettler Toledo TGA/DSC STARe System, Switzerland), and were measured as a mass residue after oxidative combustion at high temperature. Temperature and environmental conditions for the oxidative combustion of the sample were as follows: 25 ºC (10 min, N_2) – 150 ºC (3 ºC/min, N_2) – 600 ºC (10 ºC/min, Air, 60 min). The sample container was an alumina vessel of 70 μL volume. The weight of the sample vessel was accurately measured after the sixth heat treatment under the same experimental conditions. Next, the sample was placed into the sample vessel and the sample vessel was loaded into the system; then the experiment was started. After the experiment was completed, the vessel was cooled and weighted again accurately, and compared with the initial weight. The presence or absence of nonvolatile/inorganic impurities was determined from the difference between the two weights. The weight of the sample was within a range of 2 to 4 mg.

2.2.4. Analysis of volatile organic compounds

Since 17β-estradiol is a steroid hormone, it was thought that there would be very few residual organic compounds. Therefore, in this study, the existence of volatile organic compounds (VOCs) was confirmed by observing the amount of weight change at temperature below 100 °C using TGA.

2.3. Calculation of purity

The purity of 17β-estradiol, P, was calculated by Eq. (2).

$$P = [1-(P_W+P_{IN}+P_{VOC})]\cdot P_{RS} \quad (2)$$

where, P_W is the mass fraction of water in the sample, P_{IN} is the mass fraction of nonvolatiles/inorganics in the sample, P_{VOC} is the mass fraction of residual organic solvents in the sample, and P_{RS} is the mass fraction of related structure organic compounds in the sample. Normally, final purity is reported in units

of milligram per gram (mg/g).

2.4. Uncertainty evaluation

The combined standard uncertainty of 17β-estradiol purity assessment is obtained by the quadratic combination of the uncertainties associated with each contributing impurity as in Eq. (3):

$$u(P) = \sqrt{u(P_W)^2 + u(P_{IN})^2 + u(P_{VOC})^2 + u(P_{RS})^2} \quad (3)$$

where $u(P_W)$ is the standard uncertainty of water, $u(P_{IN})$ is the standard uncertainty of nonvolatile/inorganic impurities, $u(P_{VOC})$ is the standard uncertainty of residual organic solvents, and $u(P_{RS})$ is the standard uncertainty of total related structure impurities.

3. Results and Discussion

3.1. Method development

3.1.1. Measurement of total related structure impurities

In general, total related structure impurities are characterized by chromatographic analysis such as HPLC and GC with a universal detector that responds to every organic material. Methods using GC-FID and HPLC-UV are generally regarded as meeting the requirements for universal detection of organic analytes. In this study, the related structure impurities of 17β-estradiol were first analyzed by GC-FID. A higher concentration sample is preferred for purity analysis, but too high a concentration of 17β-estradiol is not suitable for GC analysis. Therefore, 17β-estradiol was dissolved in ethanol and prepared at a concentration of 1,000 mg/kg. Because 17β-estradiol is solid and has a high melting point, temperature programming in GC analysis was started at 100 ºC. The related structure impurities were mainly other steroid hormones or their precursors, so they mostly overlapped with the 17β-estradiol peak in the GC analysis. For a complete separation of the related structure impurities, the temperature programming was controlled at a very low rate. Although this experiment took a long time, it was possible to achieve a complete separation of the related structure impurities. GC chromatograms of ethanol blank and 17β-estradiol solution are shown in *Fig.* 2. Since the ethanol blank did not show any particular peak, it was confirmed that the appeared peaks due to 17β-estradiol and other impurities. Since related structure impurities peaks were attached closely to that of 17β-estradiol, the peak area of 17β-estradiol was calculated by subtraction of areas of each impurities peak from the area of the main peak. *Fig.* 3 shows the integration pattern of 17β-estradiol and all adjacent impurities. For the correction of the base line, 17β-estradiol and the adjacent impurities were integrated as one peak

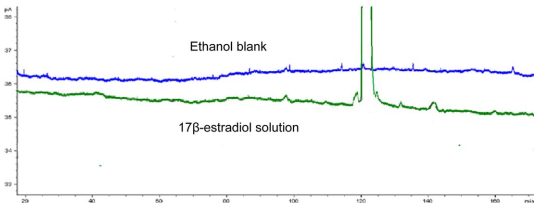

Fig. 2. GC/FID chromatograms of ethanol blank and 17β-estradiol in ethanol

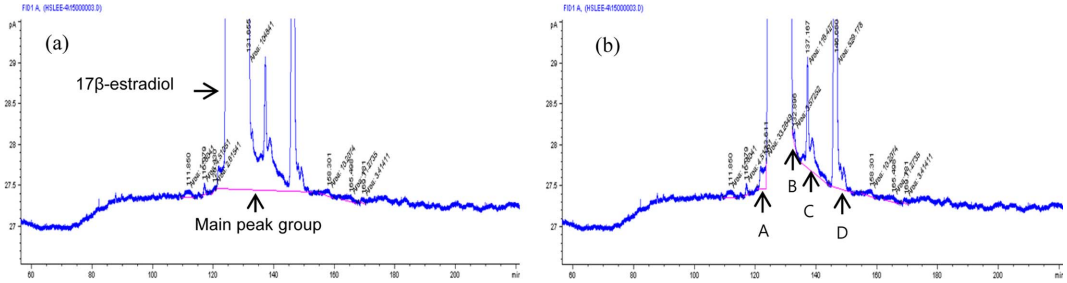

Fig. 3. Integration of 17β-estradiol in GC/FID chromatogram. (a) Integration of 17β-estradiol as a main peak group including 17β-estradiol and all adjacent impurities, (b) Integration of 17β-estradiol by subtraction of peak areas of A, B, C, and D impurities from the peak area of main peak group.

of main peak group (*Fig.* 3(a)). Next all adjacent impurities were integrated separately as shown in *Fig.* 3(b). The mass fraction of 17β-estradiol was calculated as a ratio of the peak area of 17β-estradiol to the sum of peak area of 17β-estradiol and peak areas of all related structure impurities. GC-FID experiments were repeated four times independently; the mass fraction of 17β-estradiol as determined by GC-FID was 99.3 3 %, and the standard deviation was 0.05 %, as shown in *Table* 1.

Because UV is also a universal detector responding to almost all organic compounds, for mutual comparison with GC-FID result, the related structure impurities of 17β-estradiol were also characterized by HPLC-UV method. Because17β-estradiol is a steroid hormone having a chromophore, it was possible to use the HPLC-UV method. Sample preparation was performed at a concentration of about 1,000 mg/kg in ethanol; the related structure impurities were separated by gradient elution. The gradient conditions of the mobile phase were as follows: 50 % methanol (50 min)-90 % methanol (100 min)-50 % methanol (10 min). Due to their similar structure, it was possible to completely separate 17β-estradiol and the adjacent related structure impurities by the initial isocratic of the mobile phase. It was also possible to separate other related structure impurities by gradient elution to a strong solvent. HPLC run was carried out first with the ethanol blank and next with the sample solution. No related structure impurities were found in the ethanol blank. In HPLC analysis, 17β-estradiol was completely separated from all impurity peaks as shown in *Fig.* 4. Therefore all peaks were integrated independently, and the mass fraction was calculated as a ratio of peak area of 17β-estradiol to the sum of all peak areas. The HPLC-UV experiment was carried out six times independently; the mass fraction results for 17β-estradiol by HPLC-UV are shown in *Table* 1. The mass fraction of 17β-estradiol obtained by GC-FID was 99.33 %; the value obtained by HPLC-UV was 99.27 %. The mass fraction of 17β-estradiol was set as the average value of the two values. The standard deviation of the measurement values were 0.05 % and 0.03 % respectively, showing a good reproducibility.

Table 1. Mass fraction of 17β-estradiol assigned by chromatography analysis

Measurement No.	Results by GC/FID (%)	Results by HPLC/UV (%)
1	99.35	99.32
2	99.32	99.24
3	99.37	99.28
4	99.26	99.25
5		99.23
6		99.28
Average (%)	99.33	99.27
Std. dev. (%)	0.05	0.03
Standard uncertainty (A type)	0.02	0.01
Standard uncertainty (B type)	0.12	0.15
Comb. standard uncertainty (%)	0.12	0.15
Chromatography result (%)		99.30
Standard uncertainty (%)		0.10

3.1.2. Determination of water

The water content of 17β-estradiol was determined using a Karl-Fisher Coulometer. Because 17β-

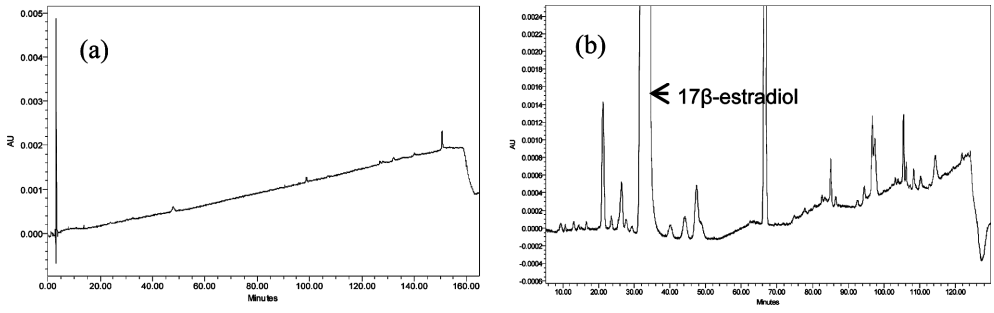

Fig. 4. HPLC/UV chromatograms of ethanol blank (a) and 17β-estradiol in ethanol (b).

estradiol is solid but soluble in methanol; the water content was determined by putting the sample directly into the reagent vessel. Since 17β-estradiol is very hygroscopic, it was inserted into the reagent vessel immediately after weighing. The weight of the sample was calculated from the weight difference between the weight of the pan containing the sample and the weight of the pan after sample insertion. The amount of water was measured in μg unit and was calculated as the ratio of the weight of water in sample to the weight of the sample. The measurement was repeated five times and the average value was used as water content. The amount of water was about 0.74 %. In order to determine the hygroscopic property of 17β-estradiol, water was measured over 4 days; the results are shown in *Table* 2. The water amounts increased in accordance with the air exposure times; thus the sample was found to be very hygroscopic as shown in *Fig.* 5. Therefore first day measurement results were used as the water content.

Table 2. Water contents measured by Karl-Fisher Coulometer

Measurement No	Water contents (%)
1	0.75
2	0.82
3	0.64
4	0.78
5	0.72
Average (%)	0.74
Std. dev. (%)	0.07
Standard uncertainty (%)	0.03

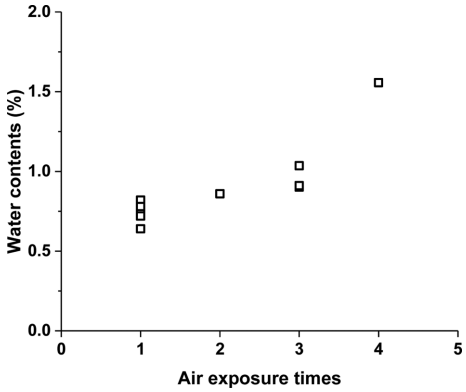

Fig. 5. Water contents change according to air exposure of 17β-estradiol.

3.1.3. Measurement of nonvolatile/inorganic impurities

A thermo gravimetric analyzer (TGA) was used for the analysis of nonvolatile/inorganic impurities. Nonvolatile/inorganic impurities comprise inorganics such as metals and metal oxides and non-volatile organic materials. The vessel used in this experiment was an alumina pan; the alumina pan was six times heat-treated under the TGA experiment conditions. Then, the weight of the alumina pan was accurately measured. A sample amount of about 4 mg was put into the pan and the pan was weighed again. The weight of the sample was calculated according to the weight difference before and after the loading of the sample. Then the sample was placed in a TGA autosampler; the temperature was raised to 150 ºC at

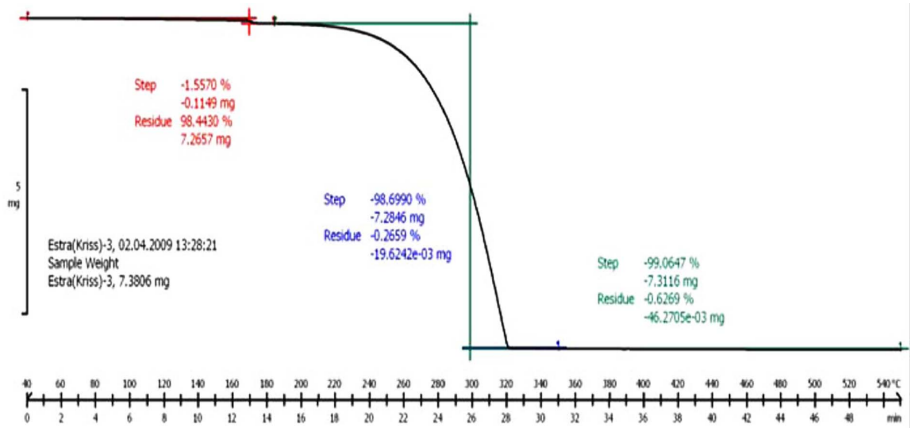

Fig. 6. Thermo gravimetric analysis result of 17β-estradiol.

a 10 ºC/min rate in nitrogen and then to 600 ºC in air at a 10 ºC/min rate. As shown in *Fig.* 6, no inflection point indicating a change in weight was observed in the nitrogen atmosphere below 150 ºC. However, in the air atmosphere above 150 ºC, 17β-estradiol was combusted with oxygen and decomposed into carbon dioxide and water with rapid weight change. But at the final temperature of 600 ºC, there was no further weight change, so only the nonvolatile/inorganic materials were considered to remain. Therefore after cooling, the pan was weighed again to measure the weight of the nonvolatile/inorganic materials. Because the weight of the alumina pan after the TGA experiment was same as its initial weight, it was found that nonvolatile/inorganic impurities were not present in the 17β-estradiol sample. Standard uncertainty was evaluated as B-type from the readability, repeatability, and linearity of analytical balance.

3.1.4. Measurement of residual organic solvents

In this study, the residual organic solvents were simultaneously checked with nonvolatiles/inorganics using TGA. Because residual organic solvents have a low boiling point, the weight change was checked at below 150 ºC. Since no apparent weight change was detected below 150 ºC in TGA graph, no residual solvent impurities were identified. As a result, the residual organic solvents were not used as an independent impurity parameter.

3.1.5. Calculation of purity

The purity of 17β-estradiol was calculated using Eq. (2); the uncertainty budget was evaluated by Eq. (3). The assigned value and the expanded uncertainty of 17β-estradiol are shown in *Table* 3. Because no nonvolatile/inorganic impurities and residual solvents were found, only the related structure impurities and water contents were included as impurities in 17β-estradiol. Therefore, the purity of 17β-estradiol was determined by the amount of total related structure organic impurities and water. The purity of 17β-estradiol was 985.6 mg/g and the expanded uncertainty was 2.1 mg/g at a 95 % confidence level.

Table 3. Results of purity assignment of 17β-estradiol

Component	Content (%)	Standard uncertainty (μ)	DOF	k	Expanded uncertainty (U)
P_{RS}	99.30 %	0.10 %	10812		
P_W	0.74 %	0.03 %	4		
P (%)	98.56 %	0.11 %	66	2	0.21
P (mg/g)	985.6	1.1	66	2	2.1

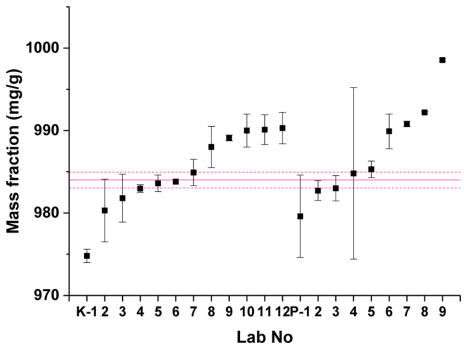

Fig. 7. Key comparison results for 17β-estradiol (Red line: key comparison reference value (KCRV), P-5: KRISS result).

3.2. Method validation by international comparison

Method validation for purity analysis of 17β-estradiol was achieved by participation in a CCQM pilot study organized by BIPM. Twenty countries participated in the key comparison; the final results are shown in *Fig.* 7. KRISS (P-5) showed result equivalent with those of other countries. Therefore, the method developed in this study for the assignment of purity of 17β-estradiol was proven to be valid and can be applied to the development of hormone CRM.

4. Conclusions

The development of a method for the purity assignment of 17β-estradiol was conducted according to the mass balance method. Impurities were classified into four classes: related structure compounds, water, nonvolatile/inorganic impurities, and residual solvents. The related structure compounds were characterized by GC-FID and HPLC/UV; water content was determined by Karl-Fisher Coulometer, and the

nonvolatile/inorganic impurities and the residual organic solvents were checked simultaneously using TGA. The developed method was validated through participation in the CCQM key comparison. The purity value of 17β-estradiol assigned by this method showed the equivalent result with small standard uncertainty among the 20 participating countries in the key comparison. International equivalence can be acquired through the key comparison, and international equivalency may provide traceability to routine measurements through certified reference material. The purity value of 17β-estradiol was 985.6 mg/g and the extended uncertainty was 2.1 mg/g.

Acknowledgements

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