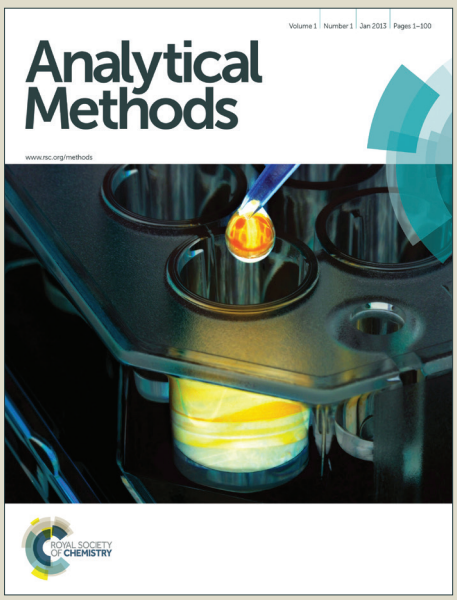
Analytical Methods

Accepted Manuscript



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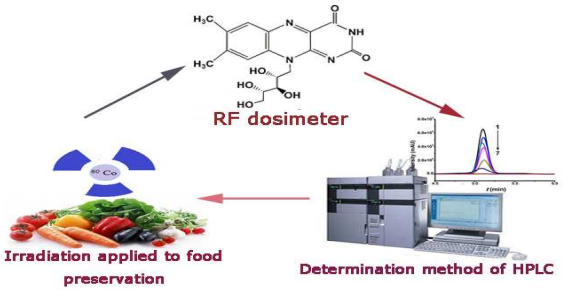
Graphical Abstract



HPLC determination method combined with riboflavin, which has sensitive irradiation characteristic, could be used in ^{60}Co dose measurement.

Analytical Methods
PAPER

Chromatographic characteristics of water-soluble vitamins with irradiation processing and its application

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This study investigated the chromatographic characteristics of irradiated vitamins and their feasibility for dose measurement by using high performance liquid chromatography (HPLC). Water soluble vitamins in B family including thiamine hydrochloride (TH), riboflavin (RF) and nicotinic acid (NA) were used for comparative study. The results showed that riboflavin was sensitive to irradiation and the content changes of RF were linearly correlated with the irradiation doses. Combining the analyses of ultraviolet and fluorescence spectra, the suitable application range for dose determination was confirmed from 100 to 2000 Gy, also, the influencing factors were further discussed. The content of RF could keep good stability before and after irradiation under low-light conditions. These characteristics make it possible to be used as a material for dose measurement. Besides, this work also provides references for irradiation nutrition research due to the favourable separation and analysis characteristics of HPLC.

Introduction

With the progress of sterilization technologies, many different physical and chemical methods have been developed, among which, ultraviolet, ozone, high temperature and autoclaving are widely applied. Generally, ultraviolet is effective in eliminating bacteria on the surface of food or space due to the weak penetrating ability. Ozone is a strong oxidizer and corrosive to material such as metal, fabric and rubber period, besides, it is stimulating and harmful to the respiration system of human operators. For many substances with unstable structures and properties, high pressure and temperature treatments are destructive factors therefore improper to be adopted. Previous studies showed that irradiation can be applied to sterilization due to the strong penetrating power and ionization effect. This technique is as simple and effective as other methods. Comparatively, irradiation consumes less energy and has been proved to be safe for health.[1] Moreover, no residue or pollutants are generated in the process and it would not influence the quality of the treated materials appreciably with appropriate dose range, these advantages make it more acceptable and prevalent. Generally, irradiation doses within 2000 Gy could inhibit the sprouting and maturation process of fruit and vegetables and thus lengthen the storage period.[2,3] Besides the applications in food preservation, it does also be used in drugs and medical apparatus as well.[4-7] With its development in the past 60 years, it has become widely applied and turned out to be well worth of practice.

The most commonly used methods for ionizing radiation dose measurement are silver dichromate spectrophotometry, thermoluminescence, ionization chamber dosimetry and color film dosimetry and so on. Among these methods, the color of dye,[8,9] radiochromic film[10] and photochromic glass[11] could change along with the different intensity of irradiation dose, but the stability and accuracy are unsatisfying. Thermoluminescence and ionization chamber dosimetry need to be connected with specialized devices, making them inconvenient to use.

Juanchi[12] studied the irradiation effect of vitamin B 12 and Maged successfully applied it to measure radiation dose in the range of 0.1-2 kGy with spectrophotometer.[13] According to their results, there was good linearity between doses and variations of vitamin concentrations in solution. In this work, a

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novel dosimeter is developed by selecting vitamins in the B family and modifying the analysis method, which proves to be more convenient for food irradiation dose measurement.

Experimental procedures

Devices and materials

Thiamine hydrochloride (99%), riboflavin (98%) and nicotinic acid (99%) were purchased from Aladdin Company (Shanghai, China). Equipments referred in this work were high performance liquid chromatograph with SPD-M 20A photodiode array detector and RF-10A fluorescence detector from Shimadzu company (Tokyo, Japan), INOVA nuclear magnetic resonance form Varian company (California, USA). The gamma radiation (γ) source employed is ^{60}Co with intensity of 5.5×10^{15} Bq, which is provided by the Application of Radiation Technology Research Center in Hunan province, China.

Irradiation process

Vitamin solutions(thiamine hydrochloride, riboflavin and nicotinic acid) with various concentrations were prepared for sampling. For selection of concentrations, our previous study showed that there was a remarkable change for TH solution of 0.05 mg/ml with 2000 Gy dose of irradiation, thus it is sensitive to this dose range and can be used. Another concentration selected in this work was 0.02 mg/ml. Water solubility of RF is poor, and it is difficult to dissolve when the concentration is above 0.05 mg/ml. Thus low concentrations of 0.025 mg/ml and 0.05 mg/ml RF solution were selected.

Niacin is diffluent in water and has good stability. Different concentrations of NA solution including 0.02 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml were employed in this work.

Vitamins of TH, RF and NA were accurately weighed and dissolved in distilled water with a volumetric flask. Amber bottles were used to prepare RF solution as it needed to be protected from light. Then these solutions were divided into the test tubes (with lids) separately. After that, these tubes were sealed up, preserved at room temperature and subjected to irradiation.

The well packaged tubes were exposed to a prescribed intensity of irradiation over certain time in order to form a 'dose'. In this experiment, the irradiation dose rate of γ ray was 1 kGy/h, and the dose range of samples accepted was from 0.1 to 2 kGy.

Results and discussion

Selection for vitamin

Vitamins are divided into water and lipid soluble variety according to their chemical characteristics. If fat-soluble ones are chosen to be dosimeter, the accuracy of the test will be interfered due to the volatilization of the organic solvent and the tendency of changing by itself upon irradiation. Thus water-soluble vitamins are more suitable to be used as the radiation mechanism of water was extensively studied in the literature.[14] It is well accepted that water could produce ·OH and H_2O_2 through the following reactions with the aid of irradiation.

$$H_2O \longrightarrow {\bullet}H + {\bullet}OH$$

$${\bullet}OH + {\bullet}OH \longrightarrow H_2O_2$$

$${\bullet}H + {\bullet}OH \longrightarrow H_2O$$

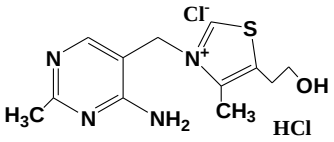
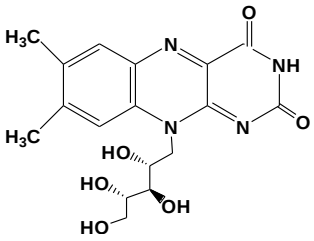
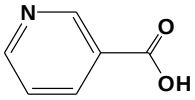
The oxidation effect of these species could destroy the structure and content of substance in water solution. Water-soluble vitamins are generally divided into vitamin B and C families. Our previous research showed that the content decrease of vitamin solution was directly related with the irradiation dose. Therefore, solutions with low concentrations are required for low dose measurements. Vitamin C is readily oxidized by γ rays owing to its oxidizable chemical structure and good solubility in water. But vitamin C solution is unstable especially at low concentrations due to hydrolysis reaction. For this reason some vitamins in B family (with brief introduction in Table 1): thiamine hydrochloride (TH), riboflavin (RF) and nicotinic acid (NA) were selected for comparative study in this work.

Content determination and dose response curve

The main apparatus used for vitamin analysis are high performance liquid chromatography (HPLC) and fluorescent spectrometry. With high accuracy and good repeatability, HPLC is widely used in purity determination of substances. Therefore in this experiment, HPLC method was employed to determine the content of the three vitamines.

The mobile phase for determination of TH and RF[15] was 0.05 mol/L sodium acetate (pH 4.5) - methanol (65 : 35), and the detection wavelength of 261 nm.[16] In this experiment, the injection volume was 20 μL, flow rate was 1 ml/min and the analytical column was Agilent

Table 1 Chemical characteristics of TH, RF and NA

No.	Biochemicals	Molecular formula	Structure	Molecular weight(g/mol)	Characteristics
1	TH	$C_{12}H_{17}ClN_4OS \cdot HCl$		337	Good stability with time, light, and temperature for thiamine combined with HCl
2	RF	$C_{17}H_{20}N_4O_6$		376	Yellow powder with fluorescent, sensitive to light and stable at relatively high temperature
3	NA	$C_6H_5NO_2$		123	Stable towards light and high temperature. No degradation produced in boiling water solution or at melting point.

TC-C_{18} (5 μm, 4.6×150 mm).

Under different irradiation doses treatment, the content variation of vitamin solutions is shown in Fig.1

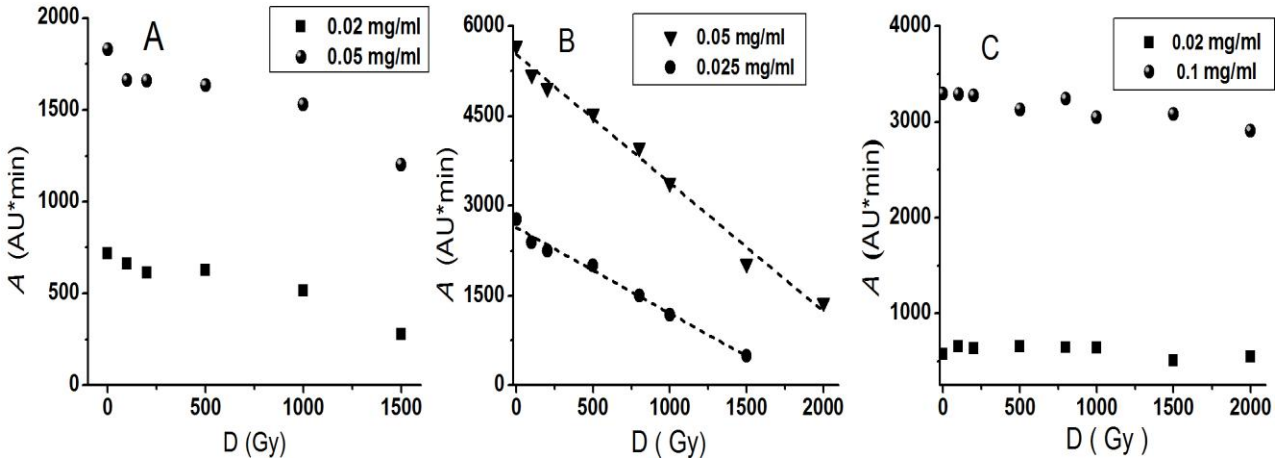


Fig.1 Scatter diagram for irradiation dose and vitamin content. (A) ρ_{TH}, 0.02 mg/ml and 0.05 mg/ml; (B) ρ_{RF}, 0.025 mg/ml and 0.05 mg/ml; (C) ρ_{NA}, 0.02 mg/ml and 0.1 mg/ml

Among the content changes of these three vitamin solutions before and after irradiation, RF was the most sensitive to γ rays, the decrease of content was obvious and the linear trend between content decrease and radiation dose was good. RF solution with concentration of 0.025 mg/ml possessed good linearity in the dose range of 100-1500 Gy, and, 0.05 mg/ml RF for 100-2000 Gy dose range, which can be applied in dose determinations with a relative coefficient both of 0.99.

Between the two concentrations of 0.02 mg/ml and 0.05 mg/ml TH, the content changes of 0.02 mg/ml was relatively obvious, but as the dose rose up to 1000 Gy, the structure of TH would be severely destroyed (with degradation occurred on main peak), which made it unsuitable for analysis. NA solutions with concentrations of 0.02 mg/ml and 0.1 mg/ml had fluctuant changes upon irradiation dose range from 100 to 2000 Gy and the total content decrease was 12 %. The content changes of 0.5 mg/ml and 1.0 mg/ml NA solutions were inconspicuous within the dose range of 2000 Gy. According to the experiments, when the dose

rose up to 4000 Gy, there were slight changes with concentration variation of 2 % and 0.9 % respectively. With high irradiation treatment from 100 to 20000 Gy, the content of 0.5 mg/ml group decreased slowly with a total change of 9.8 % and the corresponding change for 1 mg/ml NA was 4.4 %. These results showed that NA was stable upon irradiation but unsuitable for dose indication.

Comparing the content changes of the three vitamin solutions with different concentrations enduring γ radiation, the sensitivity towards irradiation decreases in the following order: riboflavin > thiamine hydrochloride > nicotinic acid. Through comparation, the content changes of RF solution had better consistency with variances of radiation doses, thus it could be used to indicate the dose changes in the range of 100-2000 Gy.

Radiation doses and spectrogram characteristics of RF

The appearance of RF solution was bright yellow and the color gradually faded as the irradiation intensity rose. The conjugated structure of RF molecular makes it good absorber of ultraviolet light, and, this characteristic can be used in quantitative analysis. Chromatogram of HPLC (Fig.2) reveals that the content of RF (with retention time at 5.23 min) continue to decline with the irradiation strengthens and the decrease shown good relevance within the range of 2000 Gy. As the residual of RF became much less, the peak of RF in the HPLC chromatogram disappeared when the dose approached 4000 Gy. Meanwhile, degradations were effectively separated shown from 1.5 min to 4 min and the total content increased with the intensified irradiation.

Besides, at an excitation wavelength of 440 nm and emission wavelength of 525 nm, RF is fluorescent which makes it easy for detection. Thus, samples that had endured irradiation were separated by HPLC system and tested by the fluorescence detector. The chromatogram variations are shown in Fig.3.

The fluorescence changing patterns of RF (with retention time at 5.20 min) were parallel to that of UV chromatogram and degradation products were detected at retention time from 1.7 to 3.7 min. As can be seen from the chromtogram, the residual of RF gradually diminished while the degradation product increased indicating that the structure of Rf molecule was destroyed by γ rays.

Riboflavin has a large conjugated plane on isoalloxazine. With the presence of free radicals

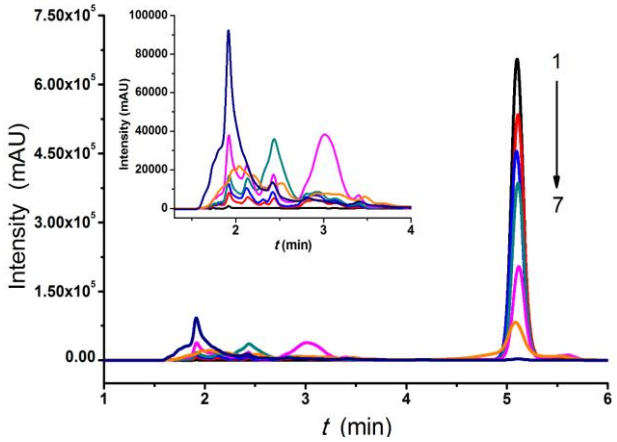


Fig.2 HPLC chromatogram of RF with different irradiation doses, curves decline from 1 to 7 denotes 0, 500, 800, 1000, 1500, 2000, 4000 Gy dose, respectively; ρ_{RF}=0.05 mg/ml

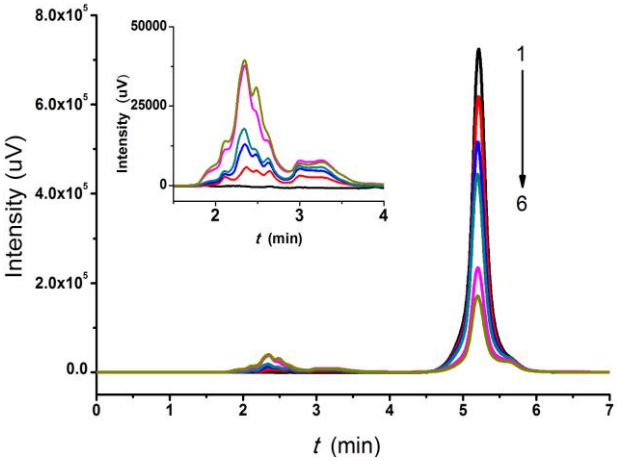


Fig.3 Fluorescence variation of RF after irradiation, curve 1 to 6 corresponds to the solutions subjected to 0, 500, 800, 1000, 1500, 2000 Gy doses respectively (samples need to be diluted for 20 times before tests)

and high energy given by γ rays, the intergrity of this constitute is easilly affected, thus qualitative change could be responsed through the sensitive spectral characteristic of RF. The structure alteration of irradiated thiamine is a seperation of thiazole and pyrimidine segment, but heterocyclic ring would not be easily affected.[17] For nicotinic acid, pyridine and formic acid are difficult to be oxidized further. As a result, RF is more sensitive towards gamma rays.

Effect of oxygen in irradiation

Oxygen is considered to be a potential factor that influences irradiation in aqueous solution. Test was carried out to examine if oxygen had obvious effect on RF irradiation system. First, 0.5 mg/ml RF solution was prepared and delivered into test tubes,

afterwards, high purity of nitrogen (99.99 %) was ventilated for 5 min in each tube. The concentration of oxygen was tested by oxygen meter. The original oxygen content was 7.1 mg/L and declined to 1.1 mg/L after nitrogen treatment. Samples were sealed up with parafilm outside the lids in order to avoid the interference of air outside. The controlling group was RF solution without nitrogen ventilating. Under the same condition, samples were exposed to irradiation and analyzed with HPLC. Results showed that there were no distinct differences either in spectrum characteristics of HPLC or content changes between the two groups, indicating that oxygen had no significant effect on this system.

Effects of temperature in dose measurement

The relations of HPLC characteristic and radiation dose at different temperatures were studied. The content of RF without irradiation remain constant under different conditions due to the stability of the RF solution. Tests revealed that there were no obvious difference at 5 ^{o}C and 20 ^{o}C. As temperature rose up to 30 ^{o}C, the residual content of RF were much lower. This indicates that radiation measurement was affected at this temperature. Therefore if the measurement is carried out at high temperatures above 30 ^{o}C, a cooling device is required.

As a result, due to different conditions of dose mesurement (sampling and analyzing), working curve needs to be calibrated to ensure the accuracy of the results.

Mechanism studies with ^{1}H-NMR spectrum

After irradiation, the content as well as spectrum characteristics of RF were changed and degradation products could be detected. In order to study the reaction mechanism of RF upon irradiation, nuclear magnetic resonance (NMR) was utilized.

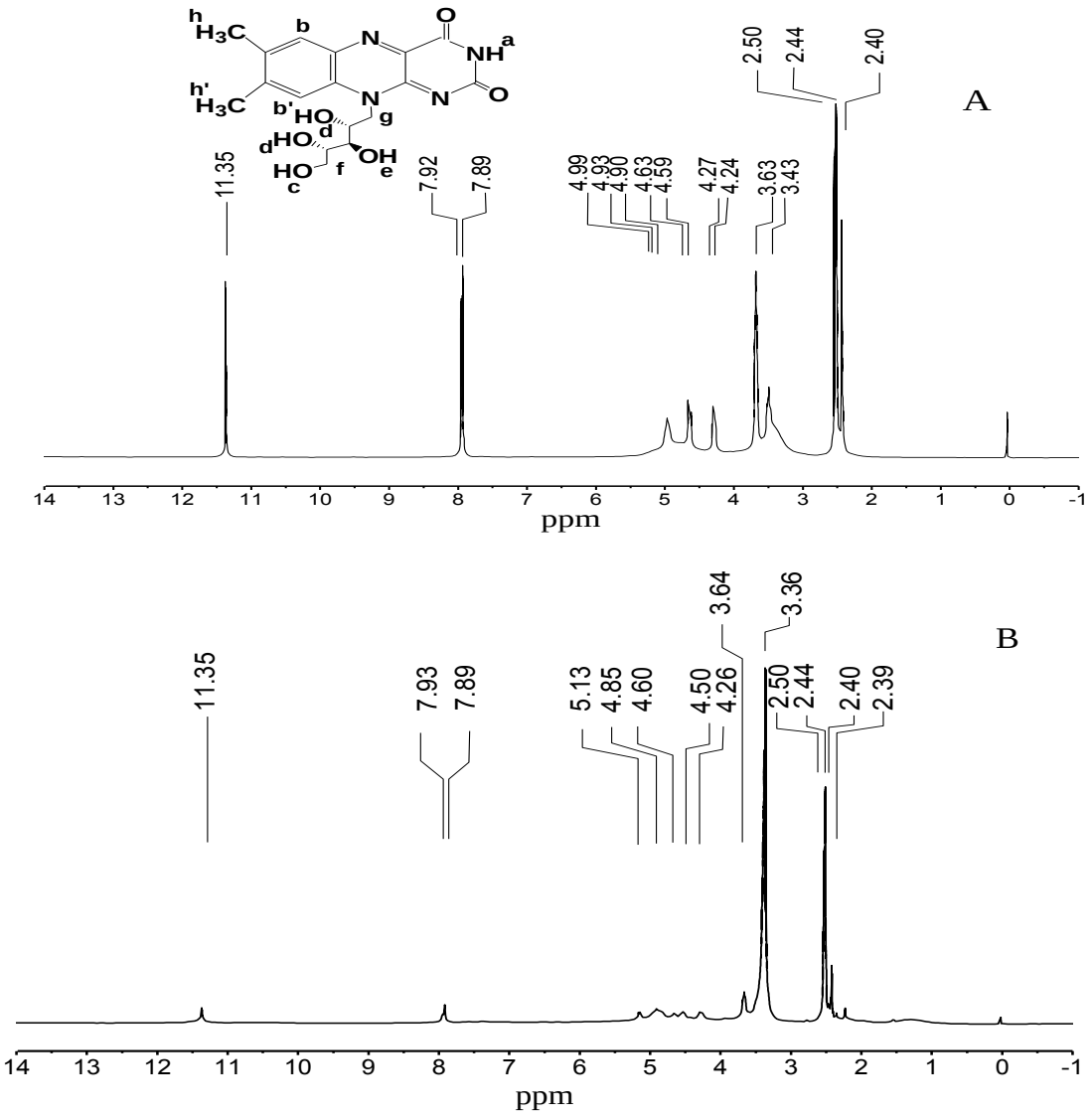


Fig.4 ^{1}H-NMR spectrum of RF unirradiated (A) and irradiated (B)

Samples of RF solution enduring irradiation and controlling group were first disposed by rotary evaporator to remove the solvent (H_2O), afterwards, they were dissolved by DMSO and tested under the same condition. ^{1}H-NMR spectrum of RF without irradiation is shown in Fig. 4 (A). The chemical shift at δ =11.35 ppm(1H, s, H-a) was assigned to proton attached to the nitrogen atom on the heterocyclic ring. The aromatic protons appeared at δ =7.92 ppm(1H, s, H-b) and δ=7.89 ppm(1H, s, H-b′). Hydrogens of hydroxyl groups were shown at δ =4.99 ppm to 4.24 ppm. The chemical shift at δ =3.63 ppm and δ =3.43 ppm corresponded to methylene(H-f, H-g) of the ribitol. δ= 2.44 ppm and δ =2.40 ppm were attributed to methyl on isoalloxazine(H-h; H-h′), which is thought to be derived from the solvent (DMSO, δ =2.50 ppm).

The spectrum of RF subjected to irradiation is shown in Fig.4 (B). Comparing these two spectra, the distribution of hydrogen remains unchanged after irradiation but the intensity at lower magnetic field decreased obviously, indicating that the integrity of RF was destroyed. The shielding effect increased while conjugative effect of ^{1}H weakened. Combining with the results of the continually decreased UV absorbance and fluorescence intensity, the conjugate planes which formed lumichrome plate was disrupted by γ radiation. The main signals of hydrogen shifted to δ 3.36 ppm and 2.44 ppm, which were primarily protons of methylene and methyl groups, implying that the structure tends to be saturated gradually. For the irradiated RF, compared with the signals at δ 11.35 ppm, δ 7.92 ppm and δ 7.89 ppm in downfield, the intensity of signals at chemical shift from δ 4.2 ppm to 5.1 ppm are relatively increased. The ratio of integral value for the two parts changed from 3/4 (unirradiated) to 3/16 (irradiated), infering that hydroxyl substituents increased. These changes illustrated that bond breakage and addition reaction occurred with the ·H and ·OH produced by γ rays.

Stability of RF solution

RF is sensitive towards light, which would cause the decrease of the content, therefore samples should be kept in weak light. When applied in irradiation measurement, in order to ensure the accuracy, a controlling group of RF solution without irradiation could be set to eliminate spontaneous attenuation. Tests showed that simple light protection (e.g. paper wrap) could eliminate the photic influence as shown in Table 2.

Table 2 Stability of 0.05 mg/ml RF solution store in weak light

Time (Day)	Content (mg/ml)	Decrease percent (%)
0	0.0500	0.00
10	0.0497	0.60
15	0.0495	1.05
20	0.0489	2.09

After irradiation, the concentration of RF in the solution as well as fluorescence intensity of RF decreased. In order to testify if it continues to degrade after irradiation, samples of RF solution were analyzed and content changes were recorded. As shown in Fig.5, the change was unconspicuous after irradiation. This further demonstrates that the degradation reaction was specially caused by the γ rays and possess stability, which would not interfere with irradiation mesurement. Chromatograph curves showed that RF solution of 0.025 mg/ml and 0.05 mg/ml had good stability. No remarkable content changes were observed within 20 days and 0.05 mg/ml RF solution gave better results. So in special occasion when samples could not be analyzed instantly by HPLC, they could be stored for a certain amount of time.

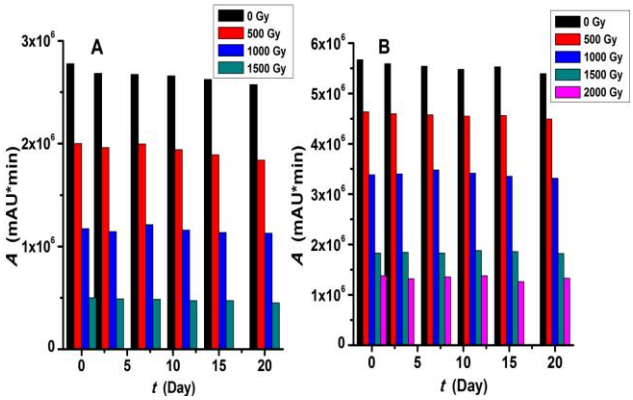


Fig.5 Stability of RF solution (0-20 Days) after irradiation, (A) 0.025 mg/ml RF solution; (B) 0.05 mg/ml RF solution

Radiation measurement

RF dosimeter was applied in irradiation field and compared with silver dichromate dosimeter by which dose distribution was calibrated. Results are shown in table 3, which indicate that dose values obtained

Table 3 Radiation dose measurement

No.	Calibrated dose (Gy)	RF Dosimeter	
		Result[1]	RSD (%)
1	100	105.27	1.7
2	500	491.69	0.9
3	1000	1001.15	0.5
4	2000	1975.73	1.1

[1]Note: Mean value of six measurements.

by this method are in good consistency with standard method.

Conclusion

Comparing the different irradiation characteristics of TH, RF and NA solutions towards γ rays, RF possesses the best linearity between concentrations variations and dose changes. With good sensitivity, selectivity and stability, 0.05 mg/ml RF solution is suitable for 100-2000 Gy irradiation dose measurement. Furthermore, the preparation for measuring is relatively simple and non-toxic, which makes it convenient to use. The accuracy of the measurement can be guaranteed by HPLC, and no other valuable equipment is needed. These merits make RF an ideal material for irradiation dose research.

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