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ORIGINAL PAPER

Study on stable radicals produced by ionizing radiation in dried fruits and related sugars by electron paramagnetic resonance spectrometry and photostimulated luminescence method – I. D-fructose

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Abstract. Stability of sugar born radicals separated from irradiated Iranian dried raisins and from D-fructose by Sigma Aldrich has been studied at room temperature by EPR and PPSL methods in a period of 360 days. It has been proven for the first time experimentally that the complex EPR spectrum of irradiated sugars is changed in time and after heating. Hence, this made it possible to distinguish spectral lines of two specific radicals contributing to the formation of multicomponent spectra, recorded in both time dependent and heating experiments. The radicals measured by EPR and energy traps detected by PPSL decay at room temperature in a similar way, suggesting a similar distribution of both species in sugar crystallites. We think that our experimental approach might be useful to study interrelation between the formation and trapping of radicals and energy accumulated in crystalline matrices. In order to achieve the isolation of individual sugars from fruits the extraction and specific separation procedures have been adapted with the use of methanol, ethanol and water solvents. Our results clearly show that radicals induced by radiation in fruits (with fructose born radicals as major constituent) are stable enough at room temperature for easy identification of irradiated raisins using the EPR method.

Key words: fructose • PPSL • EPR • irradiation • detection of • food products

Introduction

Among food articles preserved with the use of ionizing radiation one can find dried fruits containing crystalline sugar as raisins, dates, figs, mango, papaya, etc. The crystalline sugar fraction of these products is composed of fructose and glucose while other sugars appear at lower concentrations. Fructose and glucose belong to simple sugars (monosaccharides), while sucrose, for example, is disaccharide, composed of the rests of glucopyranose and fructofuranose, both connected by a 1,5-glicoside bond [1, 4].

Following the recommendation of the International Conference on the Acceptance, Control of and Trade in Irradiated Food, held in Geneva in 1988 [4] the efforts were undertaken in many countries to implement reliable methods for the detection of irradiated food suitable for its control [6, 9]. One of those method with the status of European Standard is addressed to dried fruits. The electron paramagnetic resonance (EPR) signal observed is specific, but its complexity implies multicomponent origin. The complexity of radiation induced radicals in sugars results in the uncertainty of their identification that remains not settled until now. The problem was undertaken by Belgian physicists in Ghent University who tried to evaluate from the complex spectra by means of maximum likelihood common

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Table 1. Solubility of sugars in water and in ethyl alcohol at 40°C

Sugar	Max. mass of sugar (g)	Volume of H_2O (ml)	Volume of EtOH (ml)	Concentration of final solution (%)
Glucose	208	100	–	67.5
Glucose	36	10	50	37.5
Fructose	159	50	–	76.0
Fructose	28	25	50	38.4
Sucrose	100	52	–	64.5

factor analysis (MLCFA) and density functional theory calculation (DFT) methods the number of radicals that could be expected to be formed in different sugars exposed to radiation. According to them, in α-D-glucose and β-D-fructose two, while in sucrose three, radical forms are responsible for the complexity of observed EPR spectra [8, 11, 12].

Good stability and specificity of multicomponent EPR signal are the basis of the present utilization of this signal for identification of radiation exposure in nuclear accidents [3]. The EPR spectra recorded in sugars enable to detect radiation treatment in dried fruits containing crystalline sugars (CEN standard EN 13708) [2].

The aim of the present study is to search for the complexity of multicomponent EPR signals with the use of model system consisting of purified commercial sugars and sugar fraction extracted from dried fruits (raisins), both exposed to ionizing radiation. We decided to follow two different pathways of investigation. In the first one we studied the decay and possible transformation of radiation induced EPR signals recorded at different time intervals after irradiation (1 year experiment) and after increasing the temperature up to 95°C. In our earlier experiments it has been observed that the EPR signals of irradiated dates and figs, although very stable, after prolonged storage decay slowly in time [9]. It seems rational to expect that stability of individual radicals responsible for the complexity of the EPR signal in sugars due to different molecular structures will show different stability resulting in variation of the shape of recorded spectra. From the comparison of the EPR signals recorded at different stage of storage and/or heating of samples, we expect to get some information on the spectra of specific radicals.

The second experimental approach involved the luminescence in both non-irradiated and irradiated sugars and sugar containing fruits by the pulsed photo-stimulated luminescence method (PPSL). The PPSL technique makes it possible to measure the number of shallow energy traps produced by radiation in crystalline matrices. With that experiment we intended to verify whether there is any relation between radical yield and the number of shallow energy traps.

Experimental

Materials

In the present investigation three commercial sugars delivered by Sigma Aldrich were used: alpha-D(+) glucose (anhydrous, 96% purity), D(-)fructose (98% purity). Sucrose crystalline (ultra pure), as well as *Sultan raisins* class I (permitted for consumption until 05.2009, packed and distributed in Poland, the country of origin – Iran). D(+)glucose and crystalline sucrose have been used in the present study to select the best solvent for extraction of natural sugars from fruits.

Methods

Re-crystallization of sugars

All three sugars were re-crystallized by a method proposed by Belgium researchers in Ghent University [11].

According to that procedure, sugars were re-crystallized by a single, slow (at least one month) crystallization at room temperature from saturated water (sucrose) or ethyl alcohol solutions (glucose and fructose) at 40°C. Table 1 presents the data on the solubility of various sugars in water and ethyl alcohol at 40°C after one month [2, 3, 9].

Isolation of sugars from raisins

Samples of dried, commercial raisins, weight 900 g, were grinded with a laboratory mixer and divided into three portions of 300 g each. Subsequently, each portion was treated with one of the following elution solvents: demineralized water (volume 800 ml), methanol water solution ($MeOH:H_2O$ – 4:1, volume 720 ml) or an ethanol-water solution ($EtOH:H_2O$ – 5:1, volume 565 ml).

Each solution was heated at 35°C under intensive stirring during 8 h followed by storing them under isothermal conditions overnight or longer until solutions over fruit pulp showed clear brown colour. Then, the solution was treated with the use of active carbon powder at 40°C for decolourization. Next, the hot solutions were filtered 3 times on a Büchner funnel using a thin qualitative filter paper and stirred with a benthonic powder. After filtering at 40°C, the yellow clear eluent was evaporated with a vacuum rotary evaporator at 45°C. The sugar crystallization at RT lasted from 4 to 14 weeks, depending of the solvent used.

The crystalline fraction was filtered with a funnel Schott 3G2 and washed with the same solvent. The crystallites were dried at 40°C in a laboratory oven under free flow of air. Dried crystallites were sieved with a Sigma screen cup 85 ml 60 mesh to obtain the fractions of similar homogeneity.

The EPR study was focused on D-fructose only, since preliminary results obtained with D-glucose and crystalline sucrose did not deliver reproducible and satisfactory results.

Table 2. Identification of crystalline product obtained from liquid extract of raisins based on refraction coefficient

Extraction solvent	Refraction coefficient [n] at 20°C; mean of 30 measurements		Predominant product of crystallization
	Extraction product	Reference sample	
Methanol (pure for analysis)	n = 1.34761 ± 0.00003	n = 1.34645 ± 0.00003 D(+)glucose	glucose
Ethanol (95%)	n = 1.34428 ± 0.00003	n = 1.34494 ± 0.00005 D(-)fructose	fructose
Water (demineralized)	n = 1.34311 ± 0.00007	n = 1.34494 ± 0.00005 D(-)fructose	fructose

Irradiation

Portions of re-crystallized, high purity fructose, glucose and crystallites isolated from raisins were placed in Petri dishes and irradiated with 1 kGy and 3 kGy of gamma rays in a ^{60}Co gamma source, "Issledovatel" (dose rate 0.96 kGy/h) at ambient temperature.

The irradiated samples were stored in closed Petri dishes before EPR and PPSL experiments.

Electron paramagnetic resonance (EPR) measurements

Samples of ca. 100 mg were placed in EPR sample tubes of 4 mm OD (Wilmad Glass Lab) and measured with X-band Bruker ESP-300E at room temperature.

Pulsed photo-stimulated luminescence measurements (PPSL)

Samples of pure sugars and sugar extracted from raisins, weighing ca. 2 mg, were placed in the open, plastic Petri dishes with a diameter of 50 mm and measured at room temperature with a PPSL system.

Preparation of samples for the PPSL measurement followed analytical procedure included in European Standard EN 13751:2007. PPSL Irradiated Food Screening System generates luminescence within the range 650–730 nm stimulated by the pulses of infrared light [7].

Kinetics of signal decay

The EPR signal intensity was measured every 30 days for 1 year. The kinetic data are presented in Fig. 3 as a function of total intensity of the second derivative signal measured as a distance between signal maximum and minimum (the sum of positive and negative extrema of the signal) vs. time. Such simplified method of evaluating the decrease of the multicomponent EPR signals is often adapted. The intensity of PPSL signals was recorded at the same time intervals as for EPR experiments.

To study the temperature effect on the stability of EPR signals the samples were heated for 10 min at 20°C (reference temperature), 40, 60, 75, 85 and 95°C, respectively. First observation of slight spectral changes have been observed at 75°C. However, well distinguished changes of the EPR spectra have been obtained at 95°C only (Fig. 5). The EPR measurements were carried out at room temperature one day after heating. All measurements were done under the same conditions – the samples were kept closed in the EPR sample tubes or in PPSL Petri dishes and stored in the darkness at ambient temperature.

Refractometric measurements

The identification of crystalline product gained from the concentrated raisin extract was achieved by comparison of the obtained refraction coefficients [n], which were measured with the use of an automatic refractometer, Rudolph research J 357.

Results and discussion

The refraction measurements of the crystalline product separated from liquid extract of raisins (see above) gave evidence of the identity of fructose and glucose. Both were obtained with the use of three extraction solvents (methanol, ethanol, water) as shown in Table 2.

It has been found that irradiated sugar crystallites obtained from raisins by extraction with ethanol give an EPR spectrum similar to that of irradiated fructose of high purity (Fig. 1).

It is clear, therefore, that in this case the isolated sugar fraction from raisins is composed entirely of fructose. On the other hand, crystallites obtained with the use of methanol as a solvent give the EPR spectrum resembling that of irradiated glucose. Similar observation has been done by other authors [5]. When water is used as an extraction solvent, the EPR signal of sugar crystals is complex one, however the spectrum of fructose radicals predominates.

Based on quantitative EPR results, we can conclude that ethanol and water are both suitable for extraction of fructose crystallites from raisins while methanol

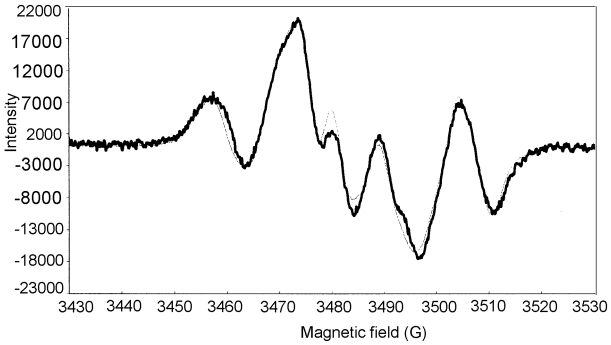

Fig. 1. EPR spectra of commercial fructose (dashed line) and the same fructose re-crystallized from EtOH (continuous line), after irradiation with a dose of 3 kGy and intensity normalization.

Table 3. Yield of sugar extraction from raisins

Solution	Predominant product of crystallization	Yield of sugar extraction*
Methanol (pure for analysis)	glucose	0.14%
Ethanol (95%)	fructose	0.87%
Water (demineralized)	fructose	0.58%

* Yield refers to the initial mass of raisins.

extracts mainly glucose from the same raisin pulp. The highest yield of sugar extraction was obtained with ethanol solvent, distinctly lower with water, while the yield of extraction process with methanol solvent was extremely low (Table 3).

Although the results in Table 1 show that water is the best solvent to dissolve sugars, our results show clearly that the yield of sugar extraction from raisins is the largest for ethanol.

The quantitative measurements of EPR signals of irradiated fructose recorded during 1 year after irradiation showed that the radicals generated radiolytically are not completely stable.

For D-fructose irradiated with doses 1 kGy and 3 kGy (Fig. 2), the decay is quite fast for the first month, when the total intensity of EPR signal decreases by about 40%. The second period is much slower and during the next 11 months the signal intensity diminishes only by 10%.

The time-dependant decay curves of PPSL signal intensity constructed for irradiated D-fructose show qualitatively similar profiles to those characterizing the decay

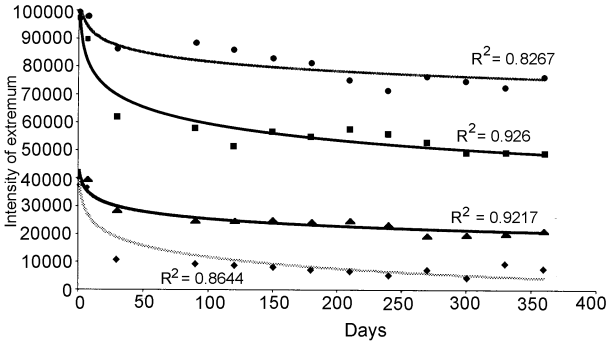

Fig. 2. Kinetic decay curves of EPR signal intensity (sum of positive and negative extrema of the signal) vs. time for irradiated fructose samples. Commercial fructose irradiated with 1 kGy (◆) and 3 kGy (■). Fructose re-crystallized and irradiated with 1 kGy (▲) and 3 kGy (●).

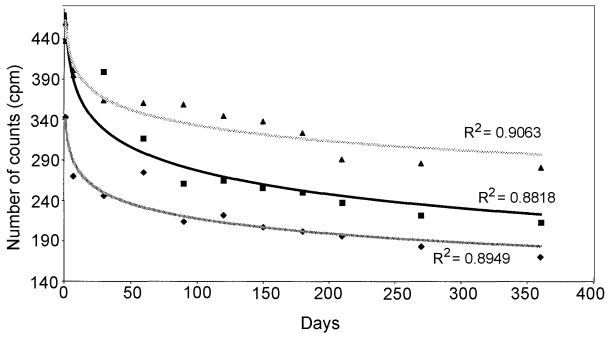

Fig. 3. Kinetic decay curves of photoluminescence (PPSL) intensity vs. time, for re-crystallized D-fructose. Re-crystallized fructose unirradiated (◆) irradiated with 1 kGy (■) and irradiated with 3 kGy (▲).

of EPR signal (Fig. 3). It can be concluded, therefore, that both radicals and energy traps are characterized by a similarly high stability in fructose matrix.

It is pertinent to note that the time-dependant decay of EPR signal intensity in commercial fructose was markedly faster if the same product has been examined after its re-crystallization. It can be deduced, therefore, that crystallites in commercial fructose are smaller (fast industrial crystallization) than those in re-crystallized fructose (slow laboratory crystallization). This influences higher stability of radicals in the latter matrix. Higher specific surface of crystallites favours faster reaction of radicals, appearing in bigger number near to the surfaces of microcrystal, for example, with the air oxygen.

From the comparison of the decay curves recorded by the EPR method (Fig. 2) and those recorded by the PPSL technique (Fig. 3), it is clearly seen that the annealing of shallow energy traps, responsible for PSL signal and decay of radicals in D-fructose, proceeds comparably slowly. A similar character of decay curves of EPR and PPSL signals of irradiated fructose suggests that radical trapping sites and energy traps in the sugar lattice are distributed in a similar way. Most probably for the fast decay are responsible the radicals and energy traps localized close to the crystallites surface, whereas more stable radicals and energy traps occupy inside crystals.

It is suggested (Von Sonntag C [13]) that in close analogy to the radiolysis of sugar in aqueous solution, radical cations generated in sugars will be deprotonated yielding oxyl radicals and protons. The oxyl radicals can undergo β-fragmentation or H-abstraction. The major part of the radicals remains immobilized in the rigid crystal matrix each other in the close vicinity. The initial relatively faster decay of radicals during the first 30 days of observation may also reflect the combination of neighbouring radicals.

In order to clear up the complex EPR spectra of irradiated sugars Callens *et al*. [10–12] developed the maximum likelihood common factor (MLCFA) model. The method was adapted to define the probable radical composition by simulating the EPR signals composed of spectra corresponding to the most probable radical structures [8, 11, 12]. It was found from that analysis that the spectra of two radicals predominate the EPR signal of irradiated D-fructose. The model derived from that method requires the removal of OH group from the carbon C-5 in the fructose molecule. In both postulated radicals unpaired electron is situated on carbon C-5 and to a smaller extent on oxygen O-5. It strongly interacts with hydrogen atom bonded with C-5 atom. Two other interactions with H atoms bonded with C-6 and C-1 carbon atoms are weaker.

In view of the similarity of principal values for both spectra Callens *et al*. suggest [11] that they probably

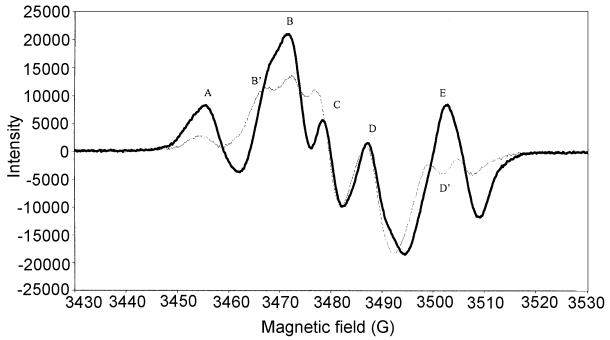

Fig. 4. The EPR spectra at 20°C of D-fructose irradiated with a dose of 1 kGy recorded 7 days (continuous line) and 360 days (dashed line) after irradiation.

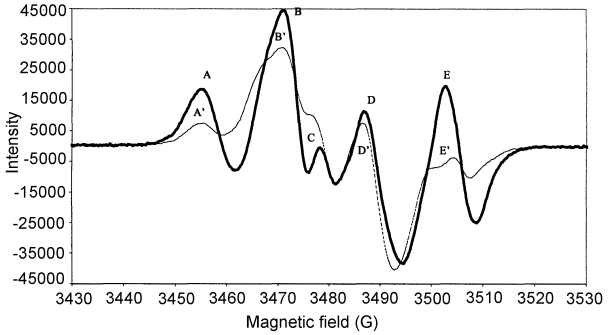

Fig. 5. The EPR spectra at 20°C of D-fructose recorded one day after irradiation with a dose of 4 kGy, then measured at 20°C (continuous line) and subsequently heated for 10 min at 95°C (dashed line).

represent the same radical trapped in two slightly different orientations with respect to crystal matrix. At present state of spectrum interpretation, the differences between two radicals are assumed to be caused by different H-bonds with the neighbouring undamaged molecules.

In spite of application of the rather sophisticated deconvolution method the authors were unable to identify radicals represented by less intensive EPR lines observed in irradiated sugars. Thus, for full understanding of radical reactions in irradiated sugars further attempts to resolve complex EPR spectra are necessary.

Our approach to that problem exploits the different stability of radiation-induced radicals. We found that after long storage at RT or heating at about 100°C of irradiated fructose some components of EPR signal decrease, making easier identification of remaining lines. The EPR spectra of γ-irradiated fructose recorded during storage at RT not only show a decrease of intensity, but also distinct spectral transformations. In Fig. 4, the normalized spectra of D-fructose recorded 7 and 360 days after irradiation are compared. This comparison clearly shows that the outer spectral lines A and E separated by 4.7 mT decrease most. In addition, the inner lines B and D, showing irregular line shape, 7 days after irradiation reveal a distinct shoulder marked in Fig. 4 as B' and D'. Those spectral changes prove that the EPR signal of irradiated fructose is composed at least of two components representing two radicals with different stability. To resolve that complex signal we tried to simulate the experimental spectrum by adding the theoretical spectra of different sugar radicals at various intensity ratios. Unfortunately, we did not produce any satisfactory result. Then, we decided to heat irradiated fructose samples expecting that this procedure would eliminate the spectral lines representing fast decaying radicals.

In Fig. 5, the EPR spectrum of irradiated D-fructose is compared with the spectrum after heating at 95°C for 10 min. EPR measurements were carried out at 20°C. The heating of the sample produced similar changes in the EPR signal as the storage for a few months at RT. In both cases the profound decrease of outer lines was observed. However, the EPR signal recorded after heating does not reveal B' and D' lines which are still visible after long-time storage. Under heating, the lines B and D are not much changed showing an asymmetric line shape. At present stage of investigation, it is not possible to assign the observed lines to the specific EPR spectra and to conclude what kind of radicals they represent. Nevertheless, it is clear that a new experimental way leading to the elucidation of this problem is opened now.

The major part of radicals remain immobilized in the crystal matrix. They can react with the neighbouring molecules when irradiated sample is dissolved in water, for example. Numerous molecular products of fructose radiolysis in solid state were identified after dissolution i.e. 2-deoxytetrose, threose, 3-deoxy-pentonic acid, arabonic acid, ribonic acid, 6-deoxy-p-threo-2,5-hexodiulose, glyceraldehyde, 3-butanone-1,2-diol, 2- and 3-deoxyhexodiulose, and molecular hydrogen, of course. It seems rational to expect that stable radical giving rise to the complex EPR spectrum of irradiated fructose are precursors of some of these products [13].

In order to understand radical reactions in irradiated sugar the complete interpretation of EPR signal recorded at room temperature would be very helpful. The first step in that direction was done by the Belgian group. Owing to the application of MLCFA method the major stable radical was postulated [8, 11, 12].

We hope that our experimental approach will be helpful to make additional progress in elucidating radical processes in irradiated sugars.

We discovered that at least one of radicals produced in fructose by radiation reveals lower stability than the others, as proven by the observation of faster decay of two well distinguished outer lines (A' and E') in multicomponent spectrum. In addition, distinct transformation of central part of the spectrum proves that spectral lines (B' and D') belong to that less stable fructose born radical, too.

At this point of our studies, we suppose that the spectrum of irradiated fructose recorded at RT may represent two types of $\bullet C_6H_{11}O_5$ radical with unpaired electron on carbon C-5 as postulated by the Belgian group [11]. The additional heating experiments are planned in order to eliminate completely the spectrum of faster decaying radical. Owing to that we should be able to reconstruct precisely the EPR spectra of major $\bullet C_6H_{11}O_5$ radicals and the spectrum of minor fructose radicals.

Conclusions

Using extraction methods we were able to achieve the isolation of sugar fraction from raisins. The sugar

fraction extracted with ethanol or with water is almost exclusively composed of fructose. Examination of the EPR spectra obtained with re-crystallized D-fructose and those of the fraction isolated with ethanol reveal almost identical shapes.

The EPR spectra of irradiated raisin fructose show significant changes during the long time storage and/or sample heating at 95°C. Those changes, reported for the first time, result from the different stability of fructose radicals generated by ionizing radiation. The decay of less stable radicals makes EPR spectra of irradiated samples simpler and will facilitate the EPR line assignment to specific radical species. Based on that method, we identified the spectra of $\bullet C_6H_{11}O_5$ radical, perhaps localized in two different matrix sites (according to fructose cyclic formula by Haward) as a major component of EPR signal of irradiated fructose. This radical is produced by the detachment of OH group from C-5 carbon, as postulated earlier [11].

Radiation induced radicals measured by EPR and energy traps released by the PPSL method decay in a similar way in both pure fructose and crystalline fructose fraction isolated from raisins. This suggests that both species are distributed in a similar way inside sugar crystallites. That observation can be a starting point to study the correlation between formation of energy traps and trapping of radicals accumulated in the lattice of irradiated sugar matrices.

Long time EPR studies on irradiated fructose show that the radicals observed at room temperature are characterized by different stabilities. That observation has been proven by thermal annealing at 95°C. Based on those experiments, we are able to conclude that the complex EPR spectrum of irradiated fructose represents at least two radicals with different stability. This is the first experimental observation proving the appearance of radicals of different stability in irradiated crystalline sugar matrix observed after long storage or heating. The experiments are in progress in order to identify those two radicals univocally.

Although the EPR spectra of fructose extracted from irradiated raisins show observable decay during the first month of examination, subsequent decay becomes markedly slower and signal intensity remains large enough to identify irradiated raisins even after a few years.

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