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Analysis of resistant starch: a method for foods and food products

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A method for resistant starch (RS) determination in food and food products is proposed. The main features are: removal of protein; removal of digestible starch; solubilization and enzymatic hydrolysis of RS; and quantification of RS as glucose released. Stomach and intestine physiological conditions (pH, transit time) were approximately simulated. All operations were performed in a 50 ml centrifuge tube. Reference materials and food products were analysed by three laboratories. Statistical analysis included repeatability and reproducibility. This procedure is quite satisfactory for starchy foods containing appreciable quantities of RS and it may be useful for nutritional labelling of foodstuffs. For samples containing $\leqslant 1\%$ RS, differences are not significant and they can be considered as foods with a negligible RS content. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Resistant starch (RS) is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (EURESTA, 1993). Raw and processed foods contain appreciable amounts of RS, depending on the botanical source of the starch and the type of processing. The amylose/amylopectin ratio, physical form, degree of gelatinization, thermal treatments, cooling and storage all affect the RS content of foods (Tovar, 1992; Sievert & Pomeranz, 1989).

The interest of nutritionists and the food industry in RS is increasing and it has led to an extensive investigation of the contribution of RS to the non-digestible carbohydrate component of the diet and its physiological implications. Colonic fermentation, bacterial growth, post-prandrial glycaemia, fecal bulking, transit time and the energy value of foods are all affected by the presence of RS (Annison & Topping, 1994; Abia *et al.*, 1993).

RS may be made up of retrograded starch, physically inaccessible starch, starch–nutrient complexes, chemically modified starch and starch that is indigestible due to enzymatic inhibition (Saura-Calixto & Abia, 1991; Englyst & Macfarlane, 1986). Retrograded starch remains in analytically determined dietary fibre (DF) residues and it contributes to an overestimation of DF content in starchy foods. A method for measuring RS in DF residues has been previously reported (Saura-Calixto *et al.*, 1993). However, the true RS content of foods is higher than that recorded in fibre residues because only a fraction of the total RS (retrograded amylose) remains in these residues.

Specific methods to determine RS in foods are needed. Direct methods quantify RS in the residues obtained after removing digestible starch (Berry, 1986; Champ, 1992). Indirect methods determine RS as the difference between total starch and digestible starch (Tovar *et al.*, 1990; Englyst *et al.*, 1992).

The aim of the present work was to develop a direct method to quantify RS in food and food products. It was derived from the Berry (1986) method with essential modifications. The main features of the analytical procedure are: removal of protein; removal of digestible starch; solubilization and enzymatic hydrolysis of RS; and quantification of RS as glucose released $\times 0.9$.

Stomach and intestine physiological conditions (pH, transit time) are approximately simulated.

MATERIALS AND METHODS

Sample preparation

Reference materials provided by the EURESTA Group were used for an interlaboratory study involving three participants. The samples were powders of bean flakes

(S1), retrograded amylose (S2), banana flour (S3), bread a (S4) and bread b (S5). Each laboratory performed eight independent analyses per sample (total: 24 determinations per sample).

Additionally, food and food products purchased in a local supermarket were analysed in laboratory 3. Raw rice, spaghetti, Corn Flakes and All Bran were directly milled to pass through a 1 mm sieve. Precooked peas and lentils were dried (air circulating oven 60°C, 24 h) and milled to obtain flours. Crispbread and biscuits were defatted after milling. Unmilled boiled rice and white bread crumbs were directly homogenized into the centrifuge tube after adding the KCl–HCl buffer.

The procedure is described in the Appendix.

Statistical analysis

The following parameters were determined according to the AOAC (Youden & Steiner, 1980): repeatability (S_r); reproducibility (S_R); repeatability relative standard deviation (RSD_r); reproducibility relative standard deviation (RSD_R). RSD_r is a measure of the within-laboratory precision and RSD_R is a measure of the values agreement among laboratories. Repeatability and reproducibility of standard deviations and coefficients of variation were calculated for each reference sample.

RESULTS AND DISCUSSION

The procedure described is based on the Berry (1986) method. The main modifications, among other minor operations, were: removal of protein; ethanol precipitation; and drying during analysis. The removal of protein was introduced to enhance amylase accessibility avoiding starch–protein associations (Lanfer-Márquez & Lajolo, 1990), starch encapsulations by protein matrix (Holm *et al.*, 1986) or formation of glutenous lumps (Batey, 1982). Moreover, this step is advisable for a better simulation of physiological conditions (proteolytic digestive enzymes, acidic pH). Depending on the sample, small but significant differences were observed in some RS values obtained with or without pepsin treatment (wheat flour: 1.63 ± 0.03, 1.93 ± 0.1; lentil flour: 8.2 ± 0.35, 8.9 ± 0.27). This proteolytic treatment was also recently introduced in the present protocol of the Englyst method (EURESTA, 1994), although this step was not included in the original procedure (Englyst *et al.*, 1992).

Ethanol precipitation and acetone washing–drying of the original Berry method come from dietary fibre analysis methodology. In RS analysis they were omitted because they are time-consuming, non-physiological conditions and drying, especially, may affect RS values.

The results from RS determinations in reference samples determined by three laboratories and the corresponding statistics are shown in Table 1. Both repeatability (S_r) and repeatability relative standard deviation (RSD_r) were considered good and gave quite close values for the three laboratories. Only four RSD_r values were higher than 10% and belonged to samples with a low RS content. However, the dispersion of the absolute values, S_r, was low. It is critical to achieve a proper homogenization of the original samples to ensure accurate sample division for analysis.

Mean values and pooled data of the three laboratories are listed in Table 2. The pooled RSD_r was 7.0%. Repeatability relative standard deviation (RSD_r) ranged from 3.3 to 23.1%, corresponding to the highest (52.0% dry matter) and the lowest (1.8% dry matter) RS content, respectively. The reproducibility relative standard deviation (RSD_R) showed lower values than RSD_r, which might indicate that this method is suitable for obtaining accurate interlaboratory data.

Statistical analyses of the food and food products (Table 3) were similar to the reference samples. The values for boiled rice (0.7%), spaghetti (1.0%), crispbread (1.0%) and All Bran (0.6%) were $\leqslant 1\%$ but without significant differences between them (ANOVA one-way).

Table 1. Resistant starch content in reference samples

	Sample	RS (% dry matter)	S_r	RSD_r (%)
Laboratory 1	S1	6.1	0.67	11.0
	S2	37.7	2.73	7.3
	S3	53.1	1.67	3.1
	S4	1.9	0.22	11.7
	S5	1.8	0.11	6.2
Laboratory 2	S1	5.9	0.10	1.6
	S2	39.1	2.62	6.7
	S3	51.8	1.66	3.2
	S4	1.9	0.26	13.5
	S5	2.2	0.13	6.0
Laboratory 3	S1	6.3	0.57	8.9
	S2	36.7	1.76	4.8
	S3	51.3	1.47	2.9
	S4	1.5	0.12	8.2
	S5	1.3	0.14	10.1

Number of independent determinations per sample: 24 (eight per laboratory). RS: resistant starch content. S_r: standard repeatability. RSD_r: repeatability relative standard deviation.

Table 2. Repeatibility and reproducibility of resistant starch analytical method

Sample	RS (% dry matter)	S_r	S_R	RSD_r (%)	RSD_R (%)
S1	6.1	0.51	0.19	8.4	3.1
S2	37.8	2.52	1.22	6.7	3.2
S3	52.0	1.72	0.95	3.3	1.8
S4	1.8	0.28	0.23	15.6	12.7
S5	1.8	0.41	0.47	23.1	26.2
Pooled	20.0	1.40	—	7.0	—

Number of independent determinations per sample: 24.RS: resistant starch content. S_r: standard repeatability. S_R: standard reproducibility. RSD_r (%): repeatability relative standard deviation. RSD_R(%): reproducibility relative standard deviation.

Table 3. Resistant starch in some foods and food products

	N	RS (% dry matter)	S_r	RSD_r (%)
1. Rice	7	6.6	1.23	18.5
2. Boiled rice	8	<1[1]	0.06	8.4
3. Spaghetti	8	<1[1]	0.46	45.2
4. Biscuit[2]	8	1.8	0.79	43.1
5. White bread (crumb)	7	2.3	0.11	4.9
6. Crispbread[2]	12	<1[1]	0.20	21.0
7. Pea flour	8	10.7	0.18	1.7
8. Lentil flour	7	8.2	0.77	9.4
9. Corn Flakes	7	3.3	0.12	3.7
10. All Bran	8	<1[1]	0.12	19.7
Pooled[3]	80	(3.4)	0.53	15.4
Pooled reduced[4]	49	(5.5)	0.68	12.2

[1]Resistant starch content lower than 1% with not significant difference.
[2]Defatted.
[3]Statistical total study.
[4]Statistical reduced study (without product numbers 2, 3, 6 and 10).

Table 4. Suggested classification of materials according to the range of resistant starch content (% dry matter)

Negligible (≤1%)
- Boiled potato (hot)
- Boiled rice (hot)
- Pasta
- Breakfast cereal containing a high proportion of bran
- Wheat flour

Low (1–2.5%)
- Breakfast cereals
- Biscuits
- Bread
- Pasta
- Boiled potato (cool)
- Boiled rice (cool)

Intermediate (2.5–5.0%)
- Breakfast cereals (Corn Flakes, Rice Crispies)
- Fried potatoes
- Extruded legumes

High (5.0–15%)
- Cooked legumes (lentils, chick peas, beans)
- Peas
- Raw rice
- Autoclaved and cooled starches (wheat, potato, maize)
- Cooked and frozen starchy foods

Very high (>15%)
- Raw potatoes
- Raw legumes
- Amylo-maize
- Unripe banana
- Retrograded amylose

In summary, this direct method is satisfactory for starchy foods containing appreciable quantities of RS. For samples containing ≤1% RS, differences are not significant and these can be considered as foods with a negligible content without any related nutritional implications.

The values obtained by indirect methods are less accurate, especially for foods with a low RS content, because they accumulate the errors of two experimental determinations. RS content of the reference samples, as determined by the Englyst method, in the EURESTA ring test were (in % dry matter): S_1, 5.14; S_2, 27.5; S_3, 53.0; S_4, 1.42; S_5, 1.78. However, the standard deviations for total starch of these standards ranged from 3.62 to 7.53 (Dysseler & Hoffem, 1994) and the accuracy of the results (total starch minus slowly digestible starch) should decrease in samples with a low RS content.

On the other hand, the direct method isolates the RS fraction, which may be used for further physico-chemical characterization or physiological tests, while for some samples only a figure is obtained by indirect methods. Englyst's procedure is probably a method more suited to determine slowly and rapidly digestible starch, than a specific method to determine RS.

After comparison between both procedures, Dysseler & Hoffem (1994) concluded that 'Berry's modified method gives the results more rapidly; it gives better and more reproducible results, with less difficulties and it is less expensive than Englyst's method. For the Englyst method, the scattering is large for low RS values. Any modification of Berry's method is preferable, especially the one of Saura-Calixto. Thus, Berry's method would be suitable for nutritional labelling of foodstuffs'.

Some chemically modified starch fragments (i.e. anhydro glucopyranoses) produced by heat treatments at low moisture may be water- and alcohol-soluble (Siljeström *et al.*, 1989), escaping from RS analysis along with oligosaccharides. Although this is a minor fraction compared with other RS types, complementary HPLC–MS analysis could be necessary for specific studies.

Table 4 shows a list of materials with the possible range of their RS contents as a guide for analysts. This classification is orientative and one must take into account that several factors, mentioned in the Introduction, could change the range given to a particular food. Some foods can be included in different groups depending on variety, ingredients and processing (i.e. pasta, breakfast cereals).

Considering that RS, like a dietary fibre, increases the indigestible matter reaching the colon, the values for foods with significant amounts of RS (such as Corn Flakes, raw rice, lentils and peas in our study) should be considered in food composition tables and food-product labelling.

Our method is at present a useful procedure to estimate RS in food and food products. However, more data are needed regarding *in vivo* experiments.

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APPENDIX: ANALYTICAL METHOD

Chemicals

- KCl–HCl buffer, pH 1.5;
- 0.1 M Tris-maleate buffer, pH 6.9, containing 4 mM $CaCl_2$;
- 0.1 M KOH;
- 0.4 M acetate buffer, pH 4.75, containing 20 mM $CaCl_2$;
- 2 M HCl;
- Pepsin (Merck No. 7190, 2000 FIT-u/G): solution containing 1 g pepsin in 10 ml KCl–HCl buffer;
- Pancreatic α-amylase (Sigma A-3176): solution containing 40 mg of α-amylase per ml of Tris-maleate buffer;
- Amyloglucosidase (Boëringer Mannheim No. 102857);
- glucose oxidase–peroxidase kit for determination of glucose (GOD/PAP, Boëhringer Mannheim No. 676543).

Procedure

Samples with low water content are milled to pass through a 1 mm sieve. If the fat content is $\geq 5\%$, samples must be defatted (petroleum–ether extraction).

When the aim of the analysis is the determination of RS in foods as eaten, drying, cooling or storage of samples must be avoided because they could affect the RS content. The samples must therefore be directly homogenized into the centrifuge tube used for analysis.

1. Weigh out 100 mg of dry milled sample into a 50-ml centrifuge tube. Add 10 ml of KCl–HCl buffer, pH 1.5. (pH adjustment with 2 M HCl or 0.5 M NaOH.) In the case of wet samples, weigh a portion equivalent to 100 mg of dry matter, add KCl–HCl buffer, pH 1.5 and homogenize into the centrifuge tube.
2. Add 0.2 ml of the pepsin solution (1 g pepsin/10 ml buffer KCl–HCl). Mix well and leave in a water bath at 40°C for 60 min with constant shaking.
3. Take samples out of the water bath and let them cool to room temperature. Add 9 ml of 0.1 M Tris-maleate buffer, pH 6.9. (pH adjustment with 2 M HCl or 0.5 M NaOH.)
4. Add 1 ml of the α-amylase solution (40 mg α-amylase per ml Tris-maleate buffer). Mix well and incubate for 16 h in a water bath at 37°C with constant shaking.
5. Centrifuge samples (15 min, 3000*g*) and discard supernatants. Wash at least once with 10 ml of distilled water, centrifuge again and discard supernatants.
6. Add 3 ml of distilled water to the residue, carefully moistening the sample. Add 3 ml of 4 M KOH, mix and leave for 30 min at room temperature with constant shaking.
7. Add approximately 5.5 ml of 2 M HCl and 3 ml of 0.4 M sodium acetate buffer, pH 4.75. (pH adjustment with 2 M HCl or 0.5 M NaOH.)
8. Add 80 μl of amyloglucosidase. Mix well and leave for 45 min in a water bath at 60°C with constant shaking.
9. Centrifuge (15 min, 3000*g*), collect supernatant and save it in a volumetric flask. Wash the residues at least once with 10 ml of distilled water, centrifuge again and combine supernatant with that obtained previously. Make up to 25–1000 ml, depending on RS content. (Alternatively, filtration of the samples could be performed instead of centrifugation.)
10. Prepare a standard curve from a glucose water solution (10–60 ppm).
11. Pipette 0.5 ml of water, sample and standard into test tubes. Add 1 ml of the reagent from the glucose determination kit (GOD–PAP). Mix well and leave for 30 min in a water bath at 37°C.
12. Read the absorbance of the samples and standards at 500 nm against a reagent blank (zero base of the spectrophotometer: reagent blank against reagent blank). Absorbances should be read between 5 and 45 min after incubation.
13. Calculations: use the standard curve to calculate the glucose concentration of the samples.
14. The resistant starch concentration of the test sample is calculated as mg of glucose $\times 0.9$.