ELSEVIER

Food Chemistry 75 (2001) 365–370

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Optimization of iron speciation (soluble, ferrous and ferric) in beans, chickpeas and lentils

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Received 18 December 2000; received in revised form 24 May 2001; accepted 24 May 2001

Abstract

A spectrophotometric method with bathophenanthroline for iron determination that makes it possible to differentiate between iron (II) and iron (III) in total soluble iron in legumes (beans, chickpeas and lentils) was optimized. Sample size, volumes of reducing agent and bathophenanthroline were selected. Matrix interferences made it necessary to apply the addition's method. To check the quality of the method, linearity and precision (RSD%) were determined. A linear response between 0.1 and 1.8 μg Fe/ml in the assay and precision values ranging from 2.1 to 6 for instrumental precision, and from 1.6 to 1.7 and 2.7 to 9.1, for intra- and inter-day assays, respectively were obtained. The application of the method to legumes indicated: total soluble iron ranging from 0.52 (microwave cooked legumes) to 5.01 mg/100 g dry matter in raw beans. The percentage of soluble iron (II) with respect to total and soluble iron ranged from non detectable to 14.8% and from non detectable to 50%, respectively.

Keywords: Beans; Chickpeas; Lentils; Legumes; Iron; Iron speciation

1. Introduction

Legumes have been and in some areas still are one of man's basic foodstuffs. From a nutritional point of view legumes are a good source of proteins, complex carbohydrates, some minerals and vitamins, and at the same time are poor in fats and sodium (Torija & Díez, 1999). The iron content of legumes together with their high consumption in different areas of the world means that they are a good source of dietary iron for large population groups.

In Spain, three species of legumes stand out for their high consumption: beans (*Phaseolus vulgaris* L.), chickpeas (*Cicer arietinum* L.) and lentils (*Lens culinaris* L.).

The nutritional value of a food of a given mineral depends not only on the mineral content, but also on its bioavailability for humans. In the case of iron, the effect that its solubility in water, oxidation state and extent of complex formation have on its bioavailability has been evaluated (Lee & Clydesdale, 1978). It is generally accepted that only soluble nonheme iron can be absorbed; thus, only a fraction of the soluble iron is available (Wienk, Marx, & Beynen, 1999). So, it is well known that iron (II) is more available than iron (III), because the latter has a low solubility in the gut. However, iron (III) can be reduced to the more soluble iron (II) in the gut by the action of gastric hydrochloric acid and reducing agents, such as ascorbic acid.

Given that only the water soluble fraction of iron is available and that the most easily absorbable sub-fraction of it is the iron (II) form, it would be useful to differentiate in legumes between soluble and insoluble iron, and in the soluble fraction between iron (II) and iron (III) (i.e. iron speciation) in order to evaluate legumes as a dietetic source of iron.

Most of the methods used to determine the iron content of foods include an organic matter destruction step that modifies the oxidation state of the element. Therefore, if we have to study the oxidation state, water extraction must be applied to extract the soluble fraction of the element. In this extract (aqueous fraction) iron (II) can be determined with bathophenanthroline (Lee & Clydesdale, 1979). The Fe(II)–tris bathophenanthroline complex has a molar absorptivity at

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0308-8146/01/$ - see front matter
PII: S0308-8146(01)00214-X

533 nm of 22 200 ± 300 (95% confidence interval) and can be extracted from the aqueous media with a non miscible solvent like isoamylic alcohol that makes it possible to concentrate the iron complex in a small volume of organic solvent (Koops, Klomp, & Elgersma, 1979).

The aim of our study was to optimize a spectrophotometric method with bathophenanthroline to determine iron and, in addition, to estimate the different oxidation states of the iron present in an aqueous extract of raw and cooked legumes, in order to estimate the availability for absorption of iron from this legumes.

2. Material and methods

2.1. Instrumentation

The following apparatuses were used: a Perkin–Elmer double beam UV–VIS spectrophotometer Lambda 2 model; a Perkin–Elmer 2380 atomic absorption spectrophotometer and a Jouan GT 422 centrifuge.

2.2. Reagents

The acetic/acetate buffer (pH 4.0) was prepared by mixing 41 ml of 0.2 M glacial acetic acid, 9 ml of 0.2 M sodium acetate and water to complete the volume to 100 ml. The bathophenantroline reagent was prepared by dissolving 120 mg of batophenantroline in 100 ml 95% v/v ethanol, and the hydroxylamine hydrochloride reducing solution by dissolving 10 g in 100 ml 50% v/v ethanol. The precipitation solution was prepared by mixing 100 g trichloroacetic acid, 100 g hydroxylamine and 100 ml hydrochloride acid (sp gr. 1.19) and adding water up to 1 l.

Iron standard solutions were prepared immediately before use by dilution of a standard solution of 1000 mg/l (Titrisol, Merck).

Chloroform, nitric acid (sp. gr. 1.40) and hydroxylamine hydrochloride were from Merck; hydrochloric acid (sp. gr. 1.19), acetic acid glacial, sodium acetate and trichloroacetic acid were from Panreac, ethanol was from Prolabo and bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) was from Sigma. All the reagents used were of analytical grade. Deionized water was used throughout (Milli Q System, Millipore).

Contamination control: in order to avoid metal contaminations glassware was washed and rinsed with water and soaked with concentrated nitric acid (sp. gr. 1.40) for 15 min. Then it was rinsed several times with distilled, deionized water.

2.3. Procedure

2.3.1. Sample treatment

The three legumes studied were: white beans (*Phaseouls vulgaris* L.), chickpeas (*Cicer arietinum* L.) and lentils (*Lens culinaris* L.). These legumes were provided by a Spanish manufacturer (ENALSA, León, Spain) as raw and ready-to-eat legumes (glass jars, highest class, weight 570 g). All analysed samples, raw and cooked, came from the same manufacturers batch. Samples were kept at room temperature until analysis.

Raw legumes (beans, chickpeas and lentils) were first ground manually with a mortar and then mechanically in an electrical mill to obtain small particles.

In order to apply cooking techniques similar to those used at home, raw legumes were soaked in tap water at room temperature for 16 h (all night) to facilitate cooking. Then the soaking water was thrown out, a volume of fresh tap water was added to the soaked legumes and they were cooked. The following legume/water ratios (w/v) were used: 1/3.8 (beans); 1/4.5 (chickpeas) and 1/2.8 (lentils). Cooking was carried out on an electric hotplate (for 45, 60 and 15 min for beans, chickpeas and lentils, respectively and in a microwave oven (1400 W) for 25 and 5 min, chickpeas and lentils, respectively. Cooked products were drained before proceeding to iron speciation.

The water content of all the raw and cooked legumes was determined so that the results could be expressed in terms of dry weight.

2.3.2. Legume extract preparation

Ten or 20 g of a ground sample of raw legumes or the equivalent amount of cooked legumes were weighed in Erlenmeyer flasks (250 ml). Sixty millilitres of deionized water was then added to the raw products while the volume added to cooked legumes was that necessary to obtain the same final weight as in the raw products. In both cases nitrogen was bubbled to remove oxygen, and the flasks were stoppered with parafilm. The whole was shaken for 5 min at room temperature and transferred to conical polyethylene tubes fitted with screwed caps. Given that the supernatant was turbid and in order to clarify it 2.5 ml of precipitant reagent was added to 5 ml aliquot of supernatant, which was then heated in a boiling water bath for 10 min. The mixture was centrifuged at 1800 ***g*** for 10 min, the supernatant (A) was used for the measuring and speciation of soluble iron.

2.3.3. Soluble iron determination

Iron (II) was determined in an aliquot of the clear supernatant by the bathophenanthroline method. To measure total soluble iron hydroxylamine chlorhydrate solution (10% w/v) was added to another aliquot to reduce iron to iron (II). The iron–bathophenanthroline complex was extracted with chloroform to remove interferences. The extract was then completed to 25 ml with 95% ethyl alcohol and centrifuged at 1800 ***g*** to obtain a clear solution. Finally, absorbance at 533 nm was measured. Simultaneously a standard calibration was carried out in the 0.1–1.6 μg iron (II)/ml range.

Different factors (weight of sample and volumes of the extract, reducing and bathophenanthroline reagents) that could affect soluble iron determination were assayed using lentils as the biological matrix.

Once the working conditions have been selected, the possible matrix effect was studied by the addition's method and then the linearity and precision of the method were determined. Finally, the selected method was applied to raw and cooked beans, chickpeas and lentils.

2.3.4. Total iron content

Simultaneously total iron content was measured by flame atomic absorption spectrometry ($\lambda = 248.3$ nm) in a solution of the ashes obtained by dry matter (d.m.) destruction at 450 °C. Citrus Leaves SRM 1572 (Standard Reference Material) NBS (National Bureau Standard, USA) were analysed together with the legumes: the obtained iron content ($\mu g\ g^{-1}$) was 95.5 ± 4.0 (certified value: 90 ± 10).

2.4. Data analysis

Statistical evaluation of the data was carried out with Statgraphics V.5.1.

The normality of the distribution and the homogeneity of the variances were tested before applying the analysis of variance (ANOVA).

To estimate the effect that the legume and cooking procedure have on the contents of soluble iron, iron (II) and iron (III), a two-way ANOVA was applied to the values obtained. Factor 1—Cooking procedure (raw, microwave, traditional and ready-to-eat). Factor 2—Legume (beans, chickpeas and lentils).

Since beans were not subjected to microwave cooking because this procedure proved inappropriate for this kind of legume, two ANOVA were applied, one including all legumes but not microwave cooking and another including only chickpeas and lentils and all cooking procedures.

Table 1
Aqueous extraction—selection of the best weight/water ratio and volume of extract for measuring soluble iron and for iron speciation

Legume	Sample (weight/water ratio)	Extract (ml)	Absorbance[a]	RSD%[b]
Lentils	10	3	0.109±0.004	3.7
	10	2	0.103±0.006	5.8
	20	1	0.017±0.009	52.9
	20	2	0.054±0.009	16.7
	20	3	0.171±0.011	6.4
Beans	10	3	0.112±0.004	3.6
Chickpeas	10	3	0.060±0.006	10.0

[a] Mean±standard deviation σ_{n-1} of four replicates.
[b] RSD, relative standard deviation.

The two-way ANOVA indicates whether or not there are interactions between the two studied factors, the type of legume and cooking procedure, that is, if one factor has an influence on the other factor. When an interaction was detected, the differences between means of the different levels of a factor were compared two by two with the minimal significant difference (MSD). A difference between them means that is higher than the MSD can be considered significant.

3. Results and discussion

3.1. Optimization of the method

The results obtained in the study of the method are reported in Tables 1, 2 and 3. Table 1 includes the values corresponding to the assays carried out to select the best weight/water ratio and volume extract for measuring soluble iron and for the iron speciation. The results obtained in the selection of the volumes of reducing solution (Table 2) and bathophenanthroline to be used are reported in Tables 2 and 3, respectively. As mentioned earlier these assays were carried out in lentils, and then the values selected for lentils were assayed in beans and chickpeas. The value of the relative standard deviation (RSD%) of the absorbance values was the criterion applied to decide the assay conditions, because the absorbance values were not comparable due to the different weight sample/water volume ratios assayed.

The best RSD% corresponded to 3 ml of an extract obtained with a sample weight/water volume ratio of 1/6 (w/v; Table 1). On the other hand, and using the extract obtained as mentioned earlier, the volumes of reducing agent and bathophenanthroline giving the

Table 2
Selection of the adequate volume of reducing agent (10% hydroxylamine hydrochloride in 50% ethanol)

Legume	Weight (g)	Reducing solution (ml)	Absorbance[a]	RSD%[b]
Lentils	10	6	0.114±0.004	3.5
		7	0.117±0.008	6.8
	20	1	0.087±0.028	32.2
		2	0.083±0.004	4.8
		3	0.144±0.008	5.6
		4	0.171±0.011	6.4
Beans	10	5	0.124±0.005	4.0
		6	0.112±0.004	3.6
Chickpeas	10	4	0.105±0.025	23.8
		5	0.145±0.011	7.6
		6	0.108±0.008	7.4

[a] Mean±standard deviation σ_{n-1} of four replicates.
[b] RSD, relative standard deviation.

most reproducible values were 6 and 5 ml, respectively (Tables 2 and 3). Therefore, the procedure to be applied for determining total soluble iron was as follows: 6 ml of reducing agent (10% hydroxylamine HCl), 1 ml buffer and 5 ml of bathophenanthroline were added to aliquots of 3 ml of the supernatant (A) and the volume was completed with deionized water to 16 ml. After shaking the solution for 1.5 min, it acquires a pink colour and immediately 10 ml of chloroform was added, the whole was shaken for 2 min and let to stand for 60 min. Then the chloroform phase was collected and the volume completed to 25 ml with 95% ethyl alcohol. After centrifuging at 1800 ***g*** for 10 min, the absorbance at 535 nm was measured in a 1-cm cell against a blank subjected to the same treatment as the sample. The same procedure was used to measure iron (II), but the reducing agent was replaced by deionized water.

Table 3
Selection of the adequate volume of reagent (0.12% batophenantroline in 95% ethanol)

Legume	Weight (g)	Batophenantroline (ml)	Absorbance[a]	RSD%[b]
Lentils	10	5	0.046±0.001	2.2
	20	1	0.024±0.005	20.8
		2.5	0.043±0.004	9.3
		5	0.054±0.009	16.7
		10	0.044±0.006	13.6
		15	0.046±0.002	4.3
Beans	10	5	0.124±0.005	4.0
		6	0.112±0.004	3.6
Chickpeas	10	4	0.134±0.010	7.5
		5	0.145±0.011	7.6

[a] Mean±standard deviation σ_{n-1} of four replicates.
[b] RSD. relative standard deviation.

Table 4
Matrix interferences study—addition's method

Set	Regression equation	Correlation coefficient	Confidence interval of slope (95%)
Aqueous standard	$y=0.0001+0.6863x$	0.9997	0.6605–0.7119
Added beans	$y=0.2433+0.4528x$	0.9999	0.4471–0.4584
Added chickpeas	$y=0.0762+0.3255x$	0.9990	0.2991–03519
Added lentils	$y=0.1534+0.05828x$	0.9992	0.3851–0.4153

3.2. Validity of the method

Once the procedure had been set up its validity was checked. Firstly, the addition's method was applied to beans, chickpeas and lentils to detect matrix interferences. A "*t*-test" was applied to compare the slopes of the regression equations corresponding to the added matrix with those of aqueous standards; differences between them indicated matrix interferences. The results obtained are reported in Table 4 and show matrix interferences in the three studied legumes. Clearly, then, the addition's method must be used to suppress the detected matrix interference.

Finally, the linearity of the response and the precision were measured by applying the earlier described procedure and the addition's method to assess the validity of the proposed method. A linear response in the 0.1–0.8 μg/ml range was obtained.

The precision values, RSDs for replicates of one extract, repeatability (intra-assays precision) and reproducibility (inter-assay precision) are reported in Table 5.

Table 5
Precision assay (RSD%)—total soluble iron and iron (II) and iron (III) (mg/100 g of d.m.) and relative standard deviation

Precision	Soluble total Fe		Soluble Fe(II)		Soluble Fe(III)	
	Content	RSD%	Content	RSD%	Content	RSD%
Extract[a]						
Beans	6.17±0.13	2.11	1.71±0.04	2.34	4.46±0.14	3.14
Chickpeas	1.81±0.11	6.07	0.47±0.04	8.51	1.33±0.13	9.77
Lentils	3.00±0.15	5.0	0.31±0.01	3.23	2.69±0.15	5.58
Repeatability[b]						
Beans	3.80±0.06	1.58	0.93±0.05	5.38	2.87±0.06	2.09
Chickpeas	1.70±0.03	1.76	0.58±0.05	8.62	1.12±0.03	2.68
Lentils	3.61±0.06	1.67	0.49±0.02	4.08	3.12±0.06	1.92
Reproducibility[c]						
Beans	3.51±0.32	9.12	0.84±0.09	10.71	2.67±0.23	8.61
Chickpeas	1.79±0.11	6.15	0.56±0.04	7.14	1.24±0.11	8.87
Lentils	3.69±0.10	2.71	0.49±0.02	4.08	3.19±0.10	3.13

[a] Relative standard deviations (RSD) for four replicates of one extract.
[b] Repeatability $n=3$.
[c] Reproducibility $n=6$.

Table 6
Total iron, soluble iron and iron (II) and iron (III) soluble in beans, chickpeas and lentils (raw, cooked and ready to eat; expressed as mg/100 g wet and d.m.)

Legume	Type	Wet matter				Dry matter			
		Fe total	Aqueous extract			Fe total	Aqueous extract		
			Fe total	Fe (II)	Fe (III)		Fe total	Fe (II)	Fe (III)
Beans	Raw	6.74±0.03	4.52±0.02	1.01±0.02	3.51±0.04	7.48±0.04	5.01±0.02 a	1.11±0.02 a	3.90±0.04
	Traditional	2.01±0.06	0.48±0.01	0.09±0.01	0.39±0.01	6.81±0.21	1.19±0.01 b	0.24±0.01 b	0.95±0.01
	Ready-to-eat	1.47±0.01	1.04±0.01	0.13±0.01	0.91±0.01	5.66±0.04	4.00±0.04 c	0.51±0.04 c	3.49±0.05
Chickpeas	Raw	4.99±0.04	1.09±0.02	0.22±0.01	0.88±0.03	5.45±0.05	1.20±0.02 a	0.24±0.02 a	0.96±0.03
	Traditional	1.44±0.03	0.23±0.01	0.12±0.01	0.11±0.01	4.41±0.09	0.69±0.01 b	0.35±0.01 b	0.34±0.02
	Microwave	1.71±0.09	0.19±0.01	0.06±0.02	0.13±0.01	4.97±0.28	0.54±0.03 c	0.18±0.01 c	0.36±0.03
	Ready-to-eat	1.11±0.01	1.08±0.02	n.d.[a]	1.08±0.02	3.53±0.01	3.42±0.07 d	n.d d	3.42±0.07
Lentils	Raw	7.75±0.08	1.91±0.01	0.20±0.18	1.71±0.01	8.60±0.09	2.12±0.01 a	0.23±0.02 a	1.89±0.01
	Traditional	2.01±0.06	0.16±0.01	0.02±0.01	0.14±0.01	7.18±0.25	0.55±0.01 b	0.06±0.01 b	0.50±0.01
	Microwave	2.25±0.07	0.15±0.01	0.01±0.01	0.14±0.01	7.76±0.25	0.52±0.01 b	0.04±0.01 b	0.48±0.01
	Ready-to-eat	1.66±0.07	1.76±0.02	0.17±0.01	1.58±0.01	5.88±0.25	6.19±0.07 c	0.60±0.03 c	5.59±0.04

The non-coincidence of letters in the same column indicates statistically significant differences ($P<0.05$). Fe(III) content was calculated by difference, subtracting Fe(II) to total soluble iron.
[a] n.d., non detectable.

This table also includes total soluble iron, soluble iron (II) and iron (III) contents of raw samples.

3.3. Total iron, soluble iron and soluble iron (II) and iron (III) of legumes

The total iron, soluble iron and iron (II) and iron (III) contents of beans, chickpeas and lentils both raw and subjected to different cooking treatments, are reported in Table 6; the results are expressed as mg/100 g dry matter.

Studies on the soluble iron content of legumes are scarce, and to our knowledge only Adewusi and Falade (1996) have studied the effect of cooking on soluble iron. These authors reported that cooking increases soluble iron contents.

From the results reported in Table 6 it is clear that total soluble iron ranged from 0.52 (microwave cooked legumes) to 5.01 mg/100 g dry matter in raw beans.

Moreover, we found, a decrease in total iron content (expressed in mg/100 g of d.m.) as a consequence of cooking at home or in the industry that can be ascribed to losses in the cooking water. No differences were detected between traditional and microwave cooking, but the ready-to-eat legumes had a lower total iron content than the home-cooked.

Logically the total soluble iron contents were lower than total iron. However, the decrease was less pronounced for the ready-to-eat legumes than for the others, and in the case of chickpeas and lentils the values were higher than those corresponding to the raw product. We believe that this is a consequence of the use of chelating agents, such as EDTA and/or citric acid, in the processing of legumes.

Only a small percentage of the soluble iron corresponds to iron (II), the most easily absorbable form.

4. Conclusions

The method reported here is useful for measuring soluble iron and iron (II) in legumes. It is essential to adopt measures in sample treatment to avoid contact with metal and oxygen, and thus prevent changes in the oxidation states of iron.

The complexity of the legume matrix and its behaviour according to the type of legume makes it necessary to apply the addition's method. It is also necessary to extract with the ferrous– bathophenanthroline complex in order to suppress interferences.

The important losses of soluble iron provoked by home-cooking processes are significantly reduced in the ready-to-eat legumes.

The percentage of soluble iron (II) with respect to total and soluble iron is very small in legumes, ranging from non detectable to 14.8% of total iron and from non detectable to 50% of total soluble iron. The iron (II) content was with one exception lower than iron (III).

Acknowledgements

This study is part of the project ALI 97/0890 financially supported by the CICYT (Spain).

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