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Optimization of heme iron analysis in raw and cooked red meat

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Abstract

Optimization of analytical conditions for heme iron extraction and its quantitative determination in raw and cooked red meat was performed by Box-Hunter design, Response Surface Methodology (RSM) was used to find a relationship between factors and response. Six analytical parameters, percent HCl in extracting solution, sample weight, stirring time, developing color time, manual stirring time, centrifugation time, were assumed to be critical factors affecting heme iron extraction. HCl concentration (X_1), sample weight (X_2), and their interaction, was the main factors affecting the effectiveness of heme extraction from meat. Optimum conditions for maximizing heme extraction were for raw meat when X_1 was 0.38 M and X_2 was 1.54 g, for cooked meat when X_1 was 0.31 M and X_2 was 1.14 g. Beef meat analyzed in this study was characterized by a high total iron content and by a corresponding high level of heme iron, representing 86% of total iron content for raw meat and 83% for cooked meat.
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Keywords: Heme iron; Heme pigment extraction; Meat

1. Introduction

Most of the literature regarding the iron content of meats and meat-based foods are generally referred to by their total iron content, few data on the heme and non-heme iron composition of meats are available. Nevertheless an accurate knowledge of the levels of the chemical forms of iron (heme, non-heme) in meat is of importance in many respects. On one hand, the reliability of the estimation of heme iron content in meat is an important task for monitoring its content in raw meats and for estimating the impact of cooking on it. Furthermore, to know the heme iron content allows the evaluation of the storage stability of meats and meat-based foods being non-heme iron, one of the major catalyst of lipid oxidation (Igene, King, Pearson, & Gray, 1979; Love & Pearson, 1974). On the other hand, meat represents the main source of available iron in the diet and a correct prediction of iron bioavailability from diets strictly depends on the careful determination of their heme iron content.

At present an official method for heme iron analysis in meat is not available. For heme iron determinations spectrophotometric methods (Chen, Chang, & Chou, 1998; Hornsey, 1956; Karlosson & Lundström, 1991), atomic absorption spectrometry methods (Kojima & Yasui, 1993), and near-infrared spectrometry methods (Hong & Yasumoto, 1996) have been developed. The Hornsey method (Hornsey, 1956) is one of the most widely used for heme pigment analysis. Literature data on heme content of red meats, however, markedly differ especially among meats characterized by high heme iron concentrations (Buchowski, Mahoney, Carpenter, & Cornforth, 1988; Carpenter & Clark, 1995; Kalpalathika, Clark, & Mahoney, 1991) differences not always attributable to the variability among species, age, and type of muscle only. Thermal processes to which foods undergo represent another important factor increasing the variability in heme iron content of meats. Heme iron in meat can be partially converted in non-heme iron by heat treatment, the type and the extent of the cooking methods strongly influence the degree of heme degradation in meat (Igene et al., 1979;

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Martinez-Tornes, Leets, Taylor, Raminez, Camacho, & Laynisse, 1986; Schricker & Miller, 1983).

This study was addressed to single out the best analytical conditions for heme iron extraction and determination in raw and cooked red meat (beef loin), when the most often method referenced (Hornsey, 1956) is used. For this purpose various analytical parameters (HCl concentration in extracting solution, sample weight, stirring initial time, developing color time in the dark, manual stirring time and centrifugation time), which can affect heme pigment extraction, were optimized as critical factors by using the Response Surface Methodology (RSM). This methodology comprises a set of techniques used in the empirical study of relationships between one or more responses and a group of input variables in order to locate the region of highest response values, where the highest is considered the best (Cornell, 1990).

2. Materials and methods

Experiments were performed on beef meat (loin) obtained by a local producer. Meat cuts were hand-trimmed of all visible fat, chopped into small pieces and divided into two equal portions. One portion was analyzed raw. The other one was cooked in an iron-free pan, stirring all the time with medium heat until meat was completely cooked and the characteristic red color of raw meat disappeared. Both raw and cooked meat samples were freeze-dried before subsequent analyses. The percentage of dry matter was determined by drying samples at 105 °C for 16 h to constant weight (AOAC, 1990). All reagents were of analytical grade and deionized water was used throughout. Glassware was acid-washed in concentrated HCl and rinsed with deionized water.

Total iron (TFe): analyses were performed by an atomic absorption spectrometer (Varian SpectrAA 40) on a graphite tube atomizer (GTA 96) under standard conditions and following liquid ashing of the samples (4 ml HNO_3 + 1 ml H_2O_2) in a microwave digestion system. Standard Reference materials: bovine muscle (BCR 184, Community Bureau of Reference, Brussels) and bovine liver (NBS 1577a, National Bureau of Standards, Gathersburg, MD 20899) were analyzed as a check on the accuracy of the analysis.

Heme iron (HFe): the analyses were carried out on freeze-dried meat samples following the analytical method described by Hornsey (1956) but critical factors such as HCl in extracting solution, sample weight, stirring initial time, developing color time in the dark, manual strirring time and centrifugation time were optimized as described in the experimental design (Table 1).

Experimental design: samples of freeze-dried beef meat (loin) were placed in a 50 ml dark capped centrifuge tube and 20 ml of an acetone solution acidified with concentrate HCl was added. The mixtures were vortex-mixed vigorously and allowed to stand in the dark and swirled by hand occasionally (the tested levels were shown in Table 1). The extracts obtained were centrifuged at 2200 rpm. The supernatants were then filtered through No. 3 Whatman filter paper and the absorbance measured at 640 nm against a reagent blank. Hematin (Sigma lot no. 57H09581) was used as standard. The HFe concentration in the samples was calculated from the standard curve and the iron content in hematin was calculated as follow:

$$\text{HFe } (\mu g/g) = \text{Hematin content } (\mu g/g) \times AW/MW$$

where AW was the atomic weight of iron and MW the molecular weight of hematin.

The optimization of the analytical conditions for heme pigment (HFe) extraction was carried out following a Box-Hunter design and the RSM was used to find the relationship between factors and response. The theorical aspects and experimental implications of RSM have been described elsewhere (Cochran & Cox, 1956). The analytical parameters tested in this study are reported in Table 1. The response-variable, HFe content, was assumed to be influenced by six independent variables (HCl in extracting solution, sample weight, stirring initial time, developing color time in the dark, manual stirring time and centrifugation time) or factors $Ø_i$ ($i = 1$–6), so that $\xi = f$ ($Ø_1$, $Ø_2$, $Ø_3$, $Ø_4$, $Ø_5$, $Ø_6$), where ξ is the response or HFe expressed as μg/g of sample, $Ø_1$ is the percent HCl in extracting solution expressed as concentration, $Ø_2$ is the sample weight expressed as g, $Ø_3$ is the stirring initial time expressed as sec, $Ø_4$ is the developing color time in the dark expressed as min, $Ø_5$ is the manual stirring time expressed as min and $Ø_6$ is the centrifugation time expressed as min. So the unknown function f was assumed to be approximate by a second degree polynomial equation such as:

Table 1
Criticals factors tested in Response Surface Methodology (RSM) analysis

Factors	Unit	Symbol		Levels		
		Coded	Uncoded	−1	0	1
HCl in extracting solution	M	X_1	ϕ_1	0.06	0.24	0.42
Sample weight	g	X_2	ϕ_2	0.66	1.10	1.54
Stirring time 1	s	X_3	ϕ_3	0	15	30
Dark time	min	X_4	ϕ_4	15	45	75
Stirring time 2	min	X_5	ϕ_5	5	10	15
Centrifugation	min	X_6	ϕ_6	5	10	15

$$\xi = b_0 + \sum_{i=l}^{k} b_i X_{\mathrm{i}} + \sum_{\mathrm{i}=1}^{\mathrm{k}} b_{ii} X_i^2 + \sum_{i=1}^{k=1} \sum_{j=2(i^4 j)}^{k} b_{\mathrm{ij}} X_{\mathrm{i}} X_{\mathrm{j}} + \varepsilon$$

where ξ is the response, b_0 (center point of system), b_{i} (coefficient of linear effects), b_{ii} (coefficient of quadratic effects) and b_{ij} (coefficient of interactive effects) are the different constant coefficients of model, X_{i} is the coded independent variable related to factors $\emptyset_{\mathrm{i}}$ and ε the error of the model. In RSM work it is advisable to

Table 2
Experimental design and response values of the six variables analyzed in both raw and cooked meat samples

Trial	Run	X_1	X_2	X_3	X_4	X_5	X_6	HFe (μg/g d.w.)		HFe (μg/g f.w.)		HFe (% TFe)	
								Raw	Cooked	Raw	Cooked	Raw	Cooked
1	15	0	−1	1	0	1	0	83.11	83.15	22.59	29.59	83.03	80.85
2	31	−1	0	0	1	1	0	57.33	65.31	15.58	23.24	57.28	63.50
3	13	0	−1	−1	0	1	0	82.60	82.33	22.44	29.30	82.52	80.05
4	49	0	0	0	0	0	0	82.85	83.76	22.51	29.81	82.77	81.44
5	52	0	0	0	0	0	0	82.09	82.49	22.31	29.36	82.01	80.21
6	11	0	−1	1	0	−1	0	84.23	82.51	22.89	29.36	84.15	80.22
7	30	1	0	0	−1	1	0	74.20	73.90	20.16	26.30	74.13	71.86
8	48	1	0	1	0	0	1	79.66	79.44	21.65	28.27	79.59	77.24
9	50	0	0	0	0	0	0	84.76	83.04	23.03	29.55	84.68	80.74
10	42	1	0	−1	0	0	−1	78.62	78.96	21.36	28.10	78.54	76.77
11	51	0	0	0	0	0	0	82.97	84.23	22.55	29.98	82.89	81.90
12	45	−1	0	−1	0	0	1	48.66	58.28	13.22	20.74	48.62	56.67
13	26	1	0	0	−1	−1	0	80.40	79.14	21.85	28.16	80.33	76.95
14	18	0	0	1	−1	0	−1	82.81	81.60	22.50	29.04	82.74	79.35
15	33	0	−1	0	0	−1	−1	89.16	87.98	24.23	31.31	89.08	85.55
16	37	0	−1	0	0	−1	1	83.17	84.72	22.60	30.15	83.09	82.37
17	54	0	0	0	0	0	0	83.02	83.14	22.56	29.59	82.94	80.84
18	29	−1	0	0	−1	1	0	49.94	56.87	13.57	20.24	49.89	55.30
19	41	−1	0	−1	0	0	−1	49.32	57.13	13.40	20.33	49.27	55.55
20	21	0	0	−1	−1	0	1	80.32	80.47	21.83	28.64	80.24	78.24
21	17	0	0	−1	−1	0	−1	80.68	81.34	21.92	28.94	80.60	79.08
22	2	1	−1	0	−1	0	0	78.35	77.82	21.29	27.70	78.28	75.67
23	22	0	0	1	−1	0	1	82.56	81.71	22.43	29.08	82.48	79.45
24	8	1	1	0	1	0	0	80.48	80.07	21.87	28.49	80.40	77.85
25	12	0	1	1	0	−1	0	81.62	82.58	22.18	29.39	81.54	80.29
26	6	1	−1	0	1	0	0	81.08	79.33	22.03	28.23	81.00	77.13
27	44	1	0	1	0	0	−1	80.90	79.02	21.98	28.12	80.82	76.83
28	27	−1	0	0	1	−1	0	60.22	68.16	16.36	24.26	60.16	66.28
29	5	−1	−1	0	1	0	0	71.01	75.64	19.30	26.92	70.95	73.54
30	23	0	0	−1	1	0	1	83.76	83.93	22.76	29.87	83.68	81.61
31	4	1	1	0	−1	0	0	76.98	78.94	20.92	28.09	76.90	76.75
32	3	−1	1	0	−1	0	0	31.35	41.29	8.52	14.70	31.32	40.15
33	14	0	1	−1	0	1	0	82.04	81.78	22.29	29.10	81.97	79.52
34	53	0	0	0	0	0	0	86.32	86.63	23.46	30.83	86.24	84.23
35	24	0	0	1	1	0	1	84.11	83.45	22.86	29.70	84.03	81.14
36	40	0	1	0	0	1	1	84.55	88.12	22.98	31.36	84.47	85.68
37	25	−1	0	0	−1	−1	0	51.69	59.84	14.05	21.30	51.64	58.19
38	47	−1	0	1	0	0	1	56.16	64.88	15.26	23.09	56.11	63.08
39	1	−1	−1	0	−1	0	0	67.26	72.15	18.28	25.68	67.19	70.16
40	19	0	0	−1	1	0	−1	83.01	84.04	22.56	29.91	82.93	81.71
41	39	0	−1	0	0	1	1	83.52	83.04	22.70	29.55	83.44	80.74
42	35	0	−1	0	0	1	−1	81.92	82.90	22.26	29.50	81.84	80.61
43	10	0	1	−1	0	−1	0	83.18	83.76	22.60	29.81	83.10	81.44
44	20	0	0	1	1	0	−1	83.97	83.52	22.82	29.72	83.89	81.21
45	43	−1	0	1	0	0	−1	55.22	64.20	15.01	22.85	55.17	62.42
46	28	1	0	0	1	−1	0	81.19	79.71	22.06	28.37	81.10	77.51
47	16	0	1	1	0	1	0	82.06	83.44	22.30	29.69	81.98	81.13
48	36	0	1	0	0	1	−1	83.65	84.03	22.73	29.90	83.57	81.71
49	7	−1	1	0	1	0	0	29.46	41.17	8.01	14.65	29.43	40.03
50	9	0	−1	−1	0	−1	0	85.12	83.31	23.13	29.65	85.04	81.00
51	46	1	0	−1	0	0	1	80.00	79.78	21.74	28.39	79.92	77.57
52	38	0	1	0	0	−1	1	83.55	83.61	22.70	29.75	83.47	81.29
53	34	0	1	0	0	−1	−1	85.14	85.62	23.14	30.47	85.06	83.25
54	32	1	0	0	1	1	0	79.93	79.34	21.72	28.23	79.85	77.14

Hfe, heme iron; Tfe, total iron.

transform natural variables into coded variables and these coded variables are usually defined as dimensionless with mean zero and the same spread or standard deviation (Myers & Montgomery, 1995). The Design Expert Software (Stat-Ease, Inc., Minneapolis) was used to generate design, regression analysis and plotting.

3. Results and discussion

The experiments were carried out on freeze-dried meats in order to perform the extraction procedure of heme pigments from samples more homogeneous than raw meats did. The use of freeze-dried samples overcame the variability intrinsic to the extractive process and due to some parameters such as type of muscolar fiber, presence of fat and differences in the moisture content of the samples. A consequence of the use of freeze-dried samples led to the introduction of a new centrifugation step in the Hornsey method (1956) which was a further variable added in the experimental design (Table 1). Table 2 shows the full experimental design and the amounts of heme iron (expressed in dry and in fresh weight) determined in both raw and cooked meats extracted under different extraction conditions. In Table 2, the "trial" column shows the order in which the experiments were carried out (a randomized order), the "run" column shows the formal or systematic order developed to obtain the experimental design. This practice must always be realised because randomization ensures that the average influence of the noise factors, such as environmental factors, is lessened (Robinson, 2000). The statistical analysis (ANOVA) of the results is reported in Table 3. Because of the high number of values of heme iron obtained for both raw and cooked samples, the standardized effect was included into Table 3 only when the effects were statistically significant ($P<0.05$). Our findings showed that among the variables tested HCl concentration (X_1) and sample weight (X_2), as well as their interaction (X_1+X_2), were the main critical factors influencing the effectiveness of heme pigments extraction in both raw and cooked meats.

Thereafter, on the basis of the application of the RSM which is a set of techniques designed to find out the best values of the responses, the best methodological conditions for heme pigments extraction were pointed out. The relationship between the two main variables (X_1 and X_2) influencing heme iron extraction was represented by the contour plot (Fig. 1). Fig. 1 showed as heme iron concentration markedly changed in function of HCl concentration and of the sample weight. In Fig. 1 the combination of the values of the two variables (X_1, X_2) which maximize the heme pigments extraction was in evidence (stationary point on the graph). These conditions were singled out for both raw (Fig. 1A) and

Table 3
Linear and quadratic effects of the six variables considered in experimental design

Model	Raw			Cooked		
	Effect	*P*	Std. Effect	Effect	*P*	Std. Effect
X_1						
Linear	27.04	0.000	13.85	18.57	0.000	10.71
Quadratic	17.32	0.000	11.63	13.47	0.000	10.28
X_2						
Linear	−7.20	0.001	−3.69	−5.039	0.006	−2.93
Quadratic	1.11	0.460		0.919	0.489	
X_3						
Linear	1.59	0.421		1.119	0.490	
Quadratic	0.80	0.591		1.168	0.380	
X_4						
Linear	3.25	0.107		3.216	0.070	
Quadratic	0.73	0.625		1.18	0.375	
X_5						
Linear	−1.98	0.317		−1.394	0.423	
Quadratic	−1.25	0.408		−1.063	0.424	
X_6						
Linear	−0.36	0.852		0.09	0.958	
Quadratic	−0.52	0.725		−0.976	0.462	
$X_1 \times X_2$	18.87	0.000	5.59	18.96	0.000	5.65

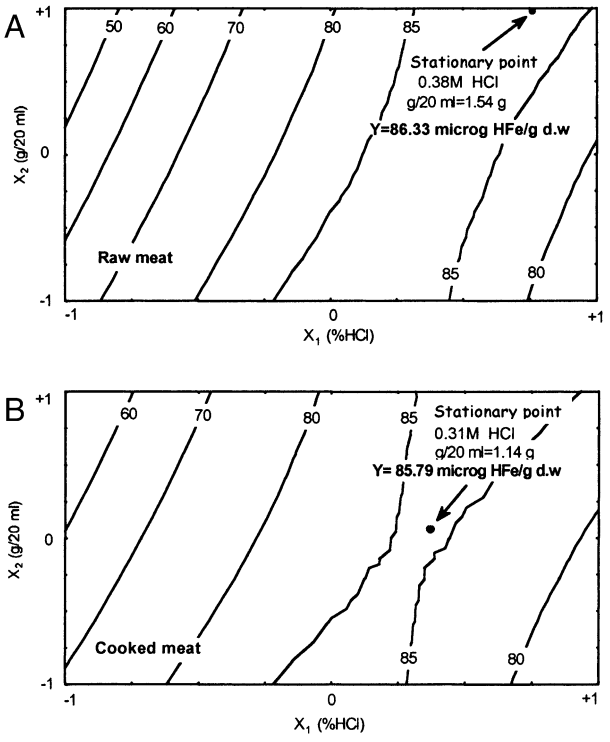

Fig. 1. Computer-generated contour plot of the estimed HFe surface, locating the stationary point for raw (A) and cooked (B) meats.

cooked (Fig. 1B) meats. The results indicated that for the two variables (X_1, X_2) the maximum of heme pigment extracting ability was obtained for raw meat when X_1 was 0.38 M and X_2 was 1.54 g. Otherwise for cooked meat the best results were obtained when X_1 was 0.31 M and X_2 was 1.14 g.

Our findings (Table 4) indicated that the beef meat (loin) analyzed in this study was characterized by a high total iron content and by a corresponding high level of heme iron, which respresented 86% of the total iron content for raw meat and 83% for cooked meat. As shown, percentage of heme iron (HFe) to total iron (TFe) decreased upon cooking, even if the loss in HFe content detected was very low (Table 4).

A large variation in total iron content of beef meat is reported in the literature (Carpenter & Clark, 1995; Igene et al., 1979; Kalpalathika et al., 1991; Schricker, Miller, & Stauffer, 1982) being that the iron concentration in meat is highly dependent on the type of cattle-breeding, age, sex and muscle of the animal (Field, Sanchez, Kunsman, & Kruggel, 1980). Available data on the percentage HFe to total iron of raw beef, obtained by different methods, varied from 61.7% to 90% (Carpenter & Clark, 1995; Kalpalathika et al., 1991). Cooking caused a reduction in percent HFe in meat depending on the heating temperature used and the time of exposure of the meat, consequently the reduction in percent HFe reported in the literature for cooked beef meat vary greatly (Carpenter & Clark, 1995; Han, McMillis, Godber, Younathan, Marshall, & Hart, 1993; Martinez-Torres et al., 1986; Rhee, Anderson, & Sams, 1996). The decrease in heme iron in meat by heat was ascribed to the oxidative cleavage of the porphyrin ring which allows the release of iron from the heme complex (Schricker et al., 1982), Han et al. (1993) showed that hemoproteins in cooked meat did not release their heme moiety during denaturation with most of the heme still associated with globin, and that an increase in heme release occurred only when temperature rose from 85 to 100 °C. In this study, very mild cooking procedures were adopted, so heating could not result in an extensive iron–porphirin complex cleavage (Table 4).

Therefore, the reasons of the strong differences in the heme iron detection among studies have to be found in the different analytical conditions adopted by the various methods and, in the case of cooked meats, also in the processing conditions utilized. The optimization of the heme iron analysis procedure performed in this study allowed the maximisation of the heme iron extraction from both raw and cooked meat. Indeed the ability of the extractive solution in the recovery of heme pigments is a key factor which can justify differences in the amounts of heme iron reported in the literature.

Table 4
Total iron (TFe), heme iron (HFe) and percent heme iron content in raw and cooked meats (f.w.)

Meat	TFe (μg/g)	HFe (μg/g)	HFe (% TFe)
Raw	27.6	23.5 (8.01–24.23)	86.29 (29.43–89.08)
Cooked	36.6	30.5 (14.65–31.36)	83.33 (40.03–85.68)

In conclusion, our results showed that the contemporary use of the Box-Hunter design and of the Response Surface Methodology allowed to identify the main variables influencing the analyses and to find out the best analytical conditions to be used in order to maximize heme pigments extraction for a quantitative determination of total heme pigments in red meats when the method of Hornsey (1965) is used.

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