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1 **Observation of the distribution of zinc protoporphyrin IX (ZPP) in Parma ham by using purple**
2 **LED and image analysis**

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1 **Abstract**

2

3 We investigated the distribution of zinc protoporphyrin IX (ZPP) in Parma ham by using
4 purple LED light and image analysis in order to elucidate the mechanism of ZPP formation.
5 Autofluorescence spectra of Parma ham revealed that ZPP was present in both lean meat and
6 fat, while red emission other than that of ZPP was hardly detected. Although ZPP was found
7 to be distributed widely in Parma ham, it was more abundant in intermuscular fat and
8 subcutaneous fat than in lean meat. The intensity of red emission was weak in muscles that
9 were exposed during the processing. ZPP in both lean meat and subcutaneous fat tended to
10 be more abundant in the inner region than in the outer region. It was thought that ZPP is
11 transferred from lean meat to fat tissue during the processing, resulting in the small amount
12 of ZPP in the lean meat adjacent to subcutaneous fat. Our results led to a completely new
13 hypothesis that ZPP is formed in lean meat and transferred to fat tissue.

14

15 **Key Words:** Parma ham, Color, Zn protoporphyrin IX, Distribution, Autofluorescence

16

1 1. Introduction

2

3 Nitrate or nitrite has recently been used in many meat products for improvement of color
4 and flavor. The characteristic red color of cured ham is caused by nitrosylmyoglobin.
5 Stable nitrosylmyoglobin is formed by the reaction of myoglobin with nitric oxide generated
6 from nitrite (Fox, 1966; Sakata, 2000).

7 Parma ham, a traditional dry-cured ham produced in the Parma region in northern Italy,
8 is made by curing the leg of a fattened pig with sea salt over a period of more than one year.
9 The addition of nitrite or nitrate to Parma ham was banned in 1993. However, the color is
10 bright red and stable. Morita, Niu, Sakata and Nagata (1996) found that an unidentified red
11 pigment, which is extractable by water or acetone/water (75%/25%), is present in Parma ham
12 and is not a myoglobin derivative such as nitrosylmyoglobin or oxymyoglobin. Møller,
13 Adamsen and Skibsted (2003a) reported that nitrosylmyoglobin was not observed in Parma
14 ham by using ESR (electron spin resonance) spectroscopy. Parolari, Gabba and Saccani
15 (2003) reported that extractability of the red Parma ham pigment by tetrahydrofuran was
16 different from that of nitrosylmyoglobin and that the pigment was more lipophilic than
17 nitrosylmyoglobin. Moreover, the pigment is stable to light and heat (Morita, Niu, Sakata &
18 Nagata, 1996; Adamsen, Møller, Hismani & Skibsted, 2004). The red pigment extracted by
19 acetone/water (75%/25%) emitted strong orange fluorescence and was identified as zinc
20 protoporphyrin IX (ZPP) by mass analysis (Wakamatsu, Nishimura & Hattori, 2004a). ZPP,
21 which is a Zn-protoporphyrin IX complex, is structurally similar to heme, which is an Fe-
22 protoporphyrin IX complex.

23 On the other hand, it has been reported that the red pigment in Parma ham is formed by
24 microorganisms, especially staphylococci (Morita, Niu, Sakata & Nagata, 1996). The
25 amount of the red pigment increases throughout the period of processing up to full
26 maturation at 18 months (Møller, Adamsen & Skibsted, 2003a). Wakamatsu, Okui, Ikeda,
27 Nishimura and Hattori (2004b) reported that ZPP is formed by anaerobic incubation of meat

1 and myoglobin in the absence of microorganisms. It has been suggested that endogenous
2 enzymes contribute to the formation of ZPP in Parma ham. However, the mechanism by
3 which ZPP is formed in Parma ham has not been elucidated.

4 Porphyrins are generally fluorescent, and red fluorescence is emitted by excitation of
5 near-ultraviolet (UV) light (about 400 nm). Some metalloporphyrins, including Zn and Mg,
6 emit strong fluorescence, but the fluorescence of most metalloporphyrins is quiescent. A
7 study on surface autofluorescence during Parma ham processing has shown that a component,
8 probably ZPP, increased in 3 months of the processing period (Møller, Parolari, Gabba,
9 Christensen & Skibsted, 2003b). Since ZPP isolated from Parma ham emitted fluorescence
10 (Wakamatsu, Nishimura & Hattori, 2004a), the distribution of ZPP could be determined by
11 detecting the fluorescence. Commercial UV lamps are generally longwave (peak wavelength:
12 365 nm) or shortwave (peak wave length: 254 nm) lamps. The optical excitation wavelength
13 of ZPP is longer than those wavelengths, being in the near-UV region (410-420 nm)
14 (Wakamatsu, Nishimura and Hattori, 2004a; Wakamatsu, Okui, Nishimura and Hattori,
15 2004b). A light-emitting diode (LED) is a semiconductor device that emits narrow-spectrum
16 light when subjected to an electric current. LED can emit ultraviolet, visible or infrared
17 light depending on the chemical composition of the semiconducting material. Thus, LEDs
18 might be a suitable light source for detecting ZPP in Parma ham.

19 The aims of the present study were to determine the macroscopic distribution of ZPP in
20 Parma ham by observation of surface autofluorescence by purple LED irradiation and to
21 obtain an insight into the mechanism by which ZPP is formed in Parma ham.

22

1 2. Materials & Methods

2

3 2.1. *Materials & sampling*

4

5 Whole Parma ham (deboned) was purchased from f.lli Galloni s.p.a.. Sliced samples of
6 approximately 2 mm in thickness were obtained by sectioning the ham using a slicer at three
7 cut lines (arrows) as shown in Fig. 1. Photographs were taken under LED irradiation in the
8 shade as much as possible within two hours after slicing.

9

10 2.2. *Purple LED lighting*

11

12 Five purple LEDs (peak wavelength: 400 nm, OSSV5111A, OptoSupply) were connected
13 in series at intervals of 10.16 mm, and nine of the LED series were connected in parallel.
14 The current was regulated by two current regulative diodes (10 mA CRD) in parallel.

15

16 2.3. *Measurement of autofluorescence spectra of Parma ham*

17

18 Autofluorescence spectra of lean meat and subcutaneous fat tissue of Parma ham
19 (approximately 1.5 cm square) were measured by using a spectrofluorophotometer
20 (RF-5300PC, Shimadzu Corp.). The excitation wavelength was 400 nm, and fluorescence
21 spectra from 450 nm to 750 nm were analyzed. Spectral bandwidths were 6 nm (excitation
22 side; half sample height) and 10 nm (emission side), and the wavelength scanning speed was
23 high mode.

24

25 2.4. *Observation of ZPP in Parma ham by purple LED lighting*

26

27 Red fluorescence of ZPP was observed by using image analysis of RAW images which

1 were taken with a digital camera (D70, Nikon Corp.). The camera was set up on a camera
2 stand in a darkroom, and slices of Parma ham were irradiated by purple LED light from two
3 directions (each angle of about 30° to the sample). The width of images was downsized to 250
4 pixels, and the colors of the images were divided in RGB channels by using Adobe Photoshop
5 6.0 (Adobe Systems Inc.). The images were saved as Microsoft Windows Bitmap Images. The
6 red emission in the R (red) channel was regarded as autofluorescence of ZPP.

7 The distribution of ZPP was compared by the average of R (red) values in each portion.
8 Tone value in the R channel was regarded as the R value (no unit). R values were obtained
9 from the downsized BMP images by using Scion Image (Beta 4.0.2, Scion Corporation), and
10 the R value in each portion was calculated by using Excel 2002 (Microsoft Corp.).
11 Densitograms were also drawn from R (red) values by using Excel 2002.

12

13 *2.5. Statistical analysis*

14

15 Data were expressed as means \pm SD. Differences among the tissues were analyzed by
16 using one-way analysis of variance (ANOVA) with Scheffé's test. Statistics were calculated
17 using Microsoft Excel 2002 and a statistic add-in software (Excel Toukei 2002 for Windows,
18 Social Survey Research Information Co., Ltd.). A probability of $P < 0.05$ was considered
19 statistically significant.

20

3. Results and Discussion

Fig. 2 shows autofluorescence spectra of Parma ham obtained by excitation at 400 nm, which is the same as the peak wavelength of the LED light used in this study. Emission peaks of lean meat were detected at 473, 593 and 632 nm, and those of subcutaneous fat tissue were detected at 467, 584, 633 and 699 nm. Since the emissions at approximately 590 and 630 nm were consistent with that of ZPP (Wakamatsu, Nishimura & Hattori, 2004a) and the emission peaks of the autofluorescence spectra of the residue extracted from Parma ham by 75% acetone had disappeared (data not shown), the emission peaks were caused by ZPP. Thus, it was shown that ZPP existed in both lean meat and subcutaneous fat tissue. ZPP tended to be more abundant in subcutaneous fat tissue than in lean meat. As shown in Fig. 2, red emission of a wavelength longer than that of ZPP (>630 nm) was hardly detected. Since the blue fluorescence at approximately 470 nm was divided by image analysis, the detection of red fluorescence due to ZPP (560-650 nm) would hardly be affected. Therefore, it was thought that the distribution of ZPP agreed with that of the red emission. On the other hand, since the fluorescence of ZPP emitted by a common fluorescence lamp (excitation wavelength: 254, 365 nm) was much weaker than that emitted by purple LED irradiation (data not shown), the LED used in this study was considered to be suitable for detection of ZPP in Parma ham.

Images of Parma ham sections irradiated with purple LED light and images of those Parma ham section images divided into R (red), G (green) and B (blue) channels are shown in Fig. 3. Purple LED images showed not only purple light of LED light origin but also red fluorescence emitted from Parma ham. After dividing into RGB channels by image analysis, green fluorescence was observed in G channel images at the circumference of sliced ham, some blood vessels and the cut section at the time of deboning. It appears to be caused by microorganisms or autofluorescence of collagen from the distribution. In B channel images, blue light of LED light origin was observed not only in Parma ham but also on the background.

1 In R channel images, red fluorescence was observed in both lean meat and fat tissues of
2 Parma ham. Since red light was not detected on the background, it was thought that the red
3 light on Parma ham was not the reflected light of LED light but probably fluorescence caused
4 by ZPP. ZPP seemed to be more abundant in the intermuscular fat and subcutaneous fat
5 than in the lean meat, in agreement with the results shown in Fig. 2. ZPP in the lean meat
6 tended to be more abundant in the inner region than in the outer region. These results show
7 that it is possible to detect the red fluorescence of ZPP origin macroscopically by using purple
8 LED light and an image analysis.

9 Densitograms were made from R values at each cross line (broken line) of red fluorescent
10 images in order to elucidate the distribution of ZPP in Parma ham (Fig. 4). Since R values of
11 subcutaneous fat (arrow) and intermuscular fat (arrow head) tended to be high, ZPP might
12 abound in fat tissue. ZPP in subcutaneous fat also tended to be more abundant in the inner
13 region than in the outer region. Curves of convex form were observed in all densitograms.
14 ZPP in lean meat tended to be more abundant in the inner region than in the outer region.
15 Since the Parma ham pigment, i.e., ZPP, is lipophylic (Møller, Adamsen & Skibsted, 2003a),
16 ZPP might be transferred from lean meat to fat tissue during the processing, resulting in the
17 small amount of ZPP in lean meat adjacent to subcutaneous fat. Further investigation of the
18 transference of ZPP, such as investigation of the solubility to fat or observation under a
19 microscope, is needed. On the other hand, the intensity of red emission was weak in
20 superficial portions of semimembranosus, gracilis and pectineus muscles (Fig. 4, asterisk in
21 section B, C); these portions were the cutting plane and were exposed during the processing of
22 ham. Since ZPP was formed under anaerobic conditions in a model system (Wakamatsu,
23 Okui, Ikeda, Nishimura & Hattori, 2004b), oxygen might inhibit the formation of ZPP.

24 Since the results from densitograms indicated that fat tissue might have a large amount
25 of ZPP, we calculated R values corresponding to ZPP contents in lean meat and fat of each
26 section (Table 1). In a preliminary experiment, the correlation coefficient between the R
27 value and the autofluorescence intensity of ZPP (Ex/Em: 420/590 nm) of Parma ham was so

1 high (0.92, $P<0.01$) that we regarded the R value as the amount of ZPP equivalent (data not
2 shown). Although a significant difference was found between ZPP contents in lean meat and
3 fat, a specific tendency was not observed. Since the standard deviation of fat was higher
4 than that of lean meat, ZPP in fat tissue was more unevenly distributed, reflecting the
5 difference in ZPP content in subcutaneous fat according to the depth from the external
6 surface. Since, however, ZPP contents in both lean meat and fat were high in the order of
7 sections B, A, and C, it is thought that ZPP did not infiltrate and diffuse from a certain
8 portion such as the cutting plane or a part of the external surface of a pork leg but formed in
9 the lean meat.

10 By further subdividing into tissues, we compared ZPP contents among muscles and fat
11 tissues (Table 2). ZPP was abundant in intermuscular fat. ZPP content of intermuscular
12 fat was significantly higher than that of the subcutaneous fat. Since the standard deviation
13 and the coefficient of variation of subcutaneous fat were high, ZPP was distributed unevenly
14 within subcutaneous fat of the same section. ZPP contents of each muscle tissue differed
15 among sections or portions, but an obvious tendency was not seen. According to the results
16 of a study on beef muscle profiling by Von Seggern, Calkins, Johnson, Brickler & Gwartney
17 (2005), the heme-iron content of the gracilis was high, the heme-iron contents of the biceps
18 femoris, pectineus and semimembranosus were nearly equal, and that of the semitendinosus
19 was low. Although the species is different from that in this study, it appears that ZPP
20 content is not directly related to heme content. However, ZPP contents in portions that were
21 exposed during the manufacturing process, especially the glacialis and pectineus (section C),
22 were low. Although the semimembranosus was also exposed during the manufacturing
23 process, ZPP contents were not so low. This is because the semimembranosus is a large
24 muscle and R values of the inner portion were high. The high coefficient of variation
25 supports this explanation. On the other hand, although the biceps femoris of section A was
26 not exposed to oxygen, ZPP content in the muscle was low. Since remarkable transference of
27 ZPP to the adjoining subcutaneous fat or intermuscular fat was not observed, ZPP might be

1 transferred within the biceps femoris.

2 ZPP has been shown to be formed by the action of microorganisms (Morita, Niu, Sakata
3 and Nagata, 1996) and in an anaerobic condition in the absence of microorganisms
4 (Wakamatsu, Okui, Ikeda, Nishimura and Hattori, 2004b), but the mechanism by which ZPP
5 is formed is still unclear. However, this study revealed that a large amount of ZPP is
6 distributed in fat tissue, where there are few heme proteins, such as myoglobin. In order to
7 elucidate the mechanism by which ZPP is formed in Parma ham, it is necessary to
8 substantiate the hypothesis that ZPP is transferred from lean meat to fat.

9

1 Conclusions

2
3 Red autofluorescence of Parma ham induced by near-UV light irradiation was derived
4 mainly from ZPP. The distribution of ZPP in Parma ham could be observed macroscopically
5 by using purple LED light and image analysis. Although ZPP was distributed widely and
6 unevenly in the interior of Parma ham, it was more abundant in fat tissue, especially
7 intermuscular fat, than in lean meat. Although the amounts of ZPP in muscles could be
8 compared relatively by using R-values, a specific tendency was not observed. Muscles
9 around the exposed portion had only a small amount of ZPP. Exposure to oxygen might have
10 inhibited the formation of ZPP in that portion. ZPP in both lean meat and subcutaneous fat
11 tended to be more abundant in the inner region than in the outer region. Our results led to a
12 completely new hypothesis that ZPP is formed in ham and is transferred from lean meat to fat
13 tissue during the processing.

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16 Acknowledgments

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1 formed in nitrite-free dry-cured ham. *Meat Science*, 68, 313-317.

2

1 Figure legends

2

3 Fig. 1. Sampling from Parma ham. Each section sample (A, B and C; about 2 mm thick)
4 was cut out from each cut line (arrows) of Parma ham. *Muscles*: SM, semimembranosus; ST,
5 semitendinosus; BF, biceps femoris; GR, gracilis; PE, pectineus; QF, quadriceps femoris; TS,
6 triceps surae.

7

8 Fig. 2. Autofluorescence spectra (excitation: 400 nm) of lean meat (solid line) and
9 subcutaneous fat tissue (broken line) in Parma ham.

10

11 Fig. 3. Detection of red ZPP fluorescence by image analysis. Autofluorescent images of
12 each Parma ham section were obtained by irradiation of purple LED light, and RGB images
13 were obtained by division of autofluorescent images into RGB channels by image analysis.

14

15 Fig. 4. Distribution of ZPP in Parma ham. Densitograms were made from tone values (R
16 values) of each cross line (broken line) in R channel images. Arrows indicate subcutaneous
17 fat and arrowheads indicate intermuscular fat. Asterisks indicate the cutting plane and its
18 environs, which were exposed during the processing.

19

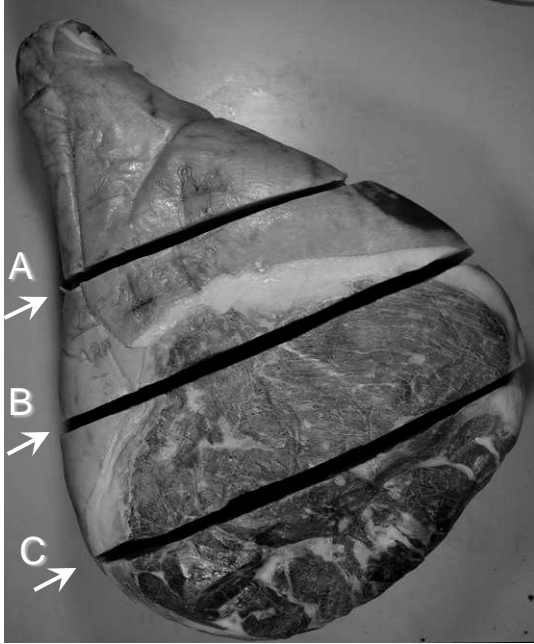
20 Table 1. Difference between ZPP contents calculated from R values of muscle and fat in each
21 Parma ham section.

22

23 Table 2. ZPP contents calculated from R values in each part of Parma ham.

24

Whole ham



Cross sections

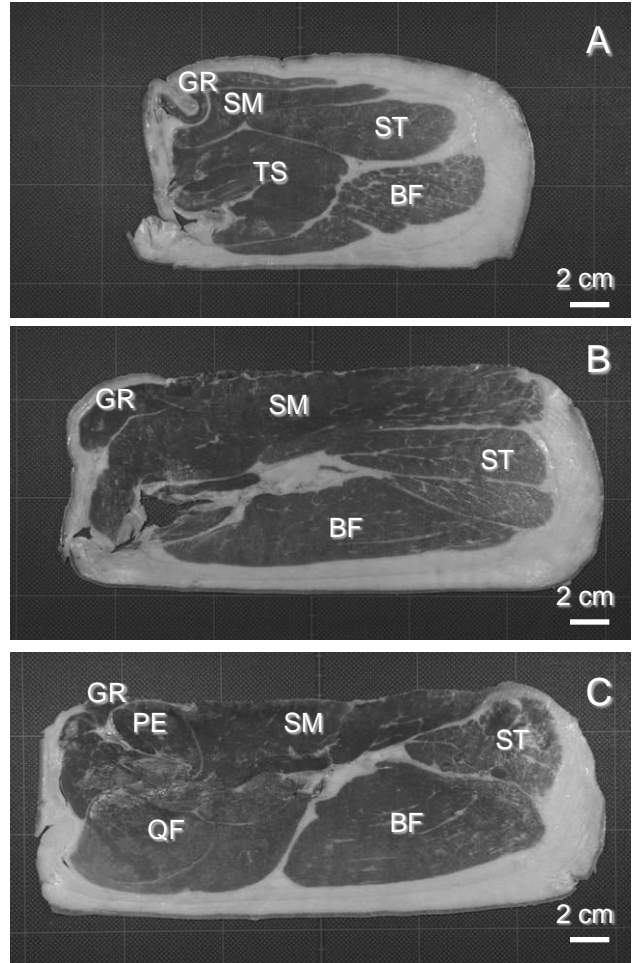


Fig. 1

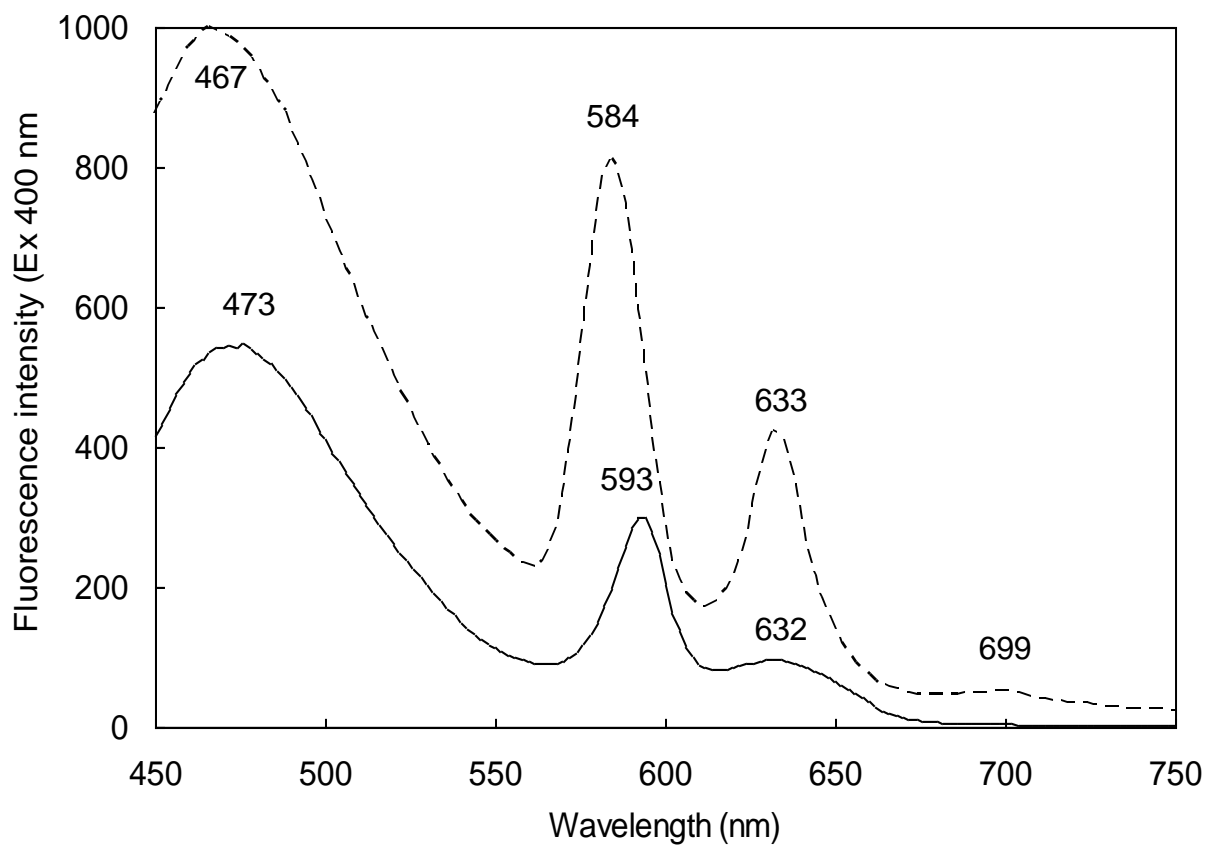


Fig. 2

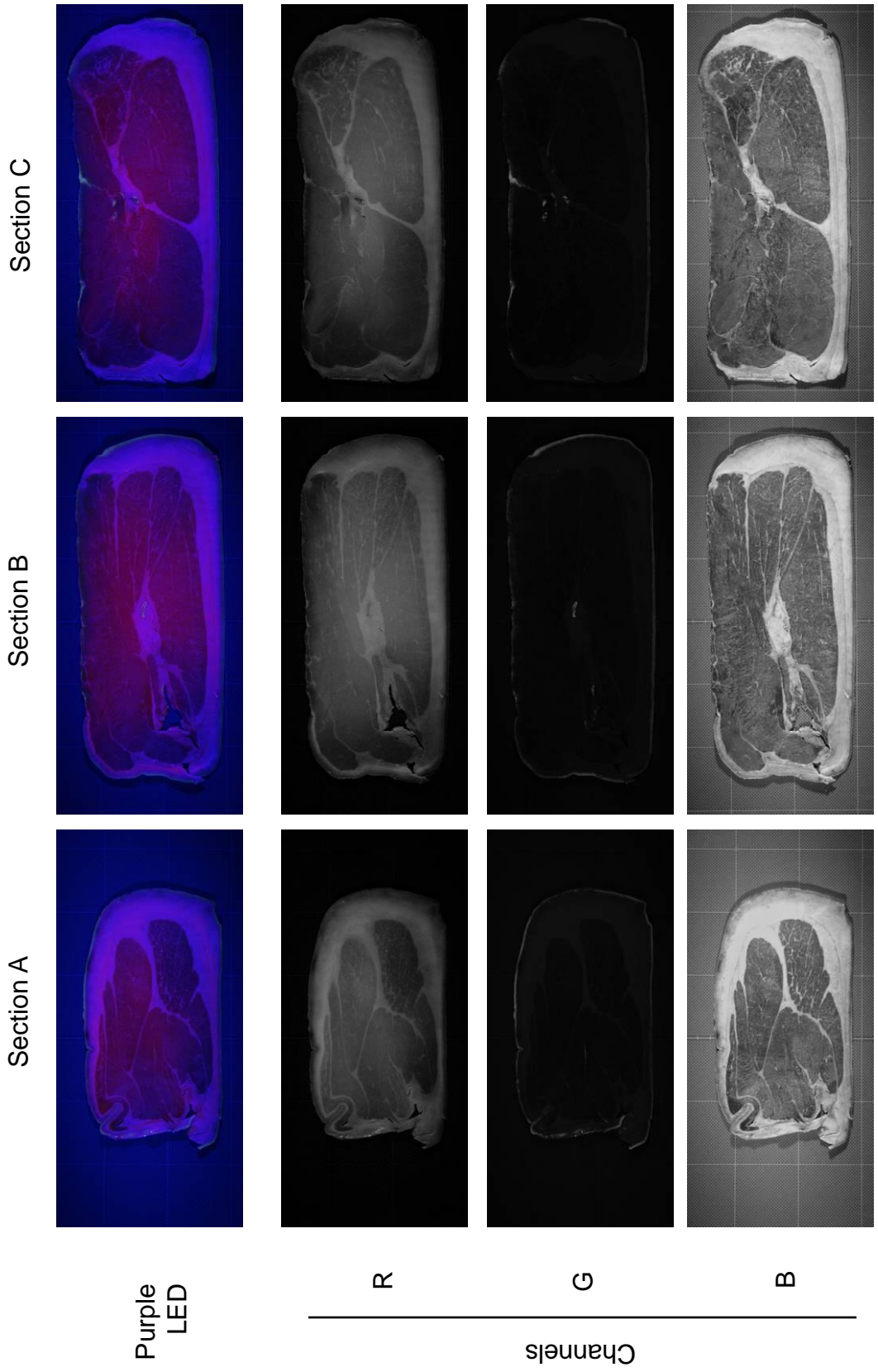


Fig. 3

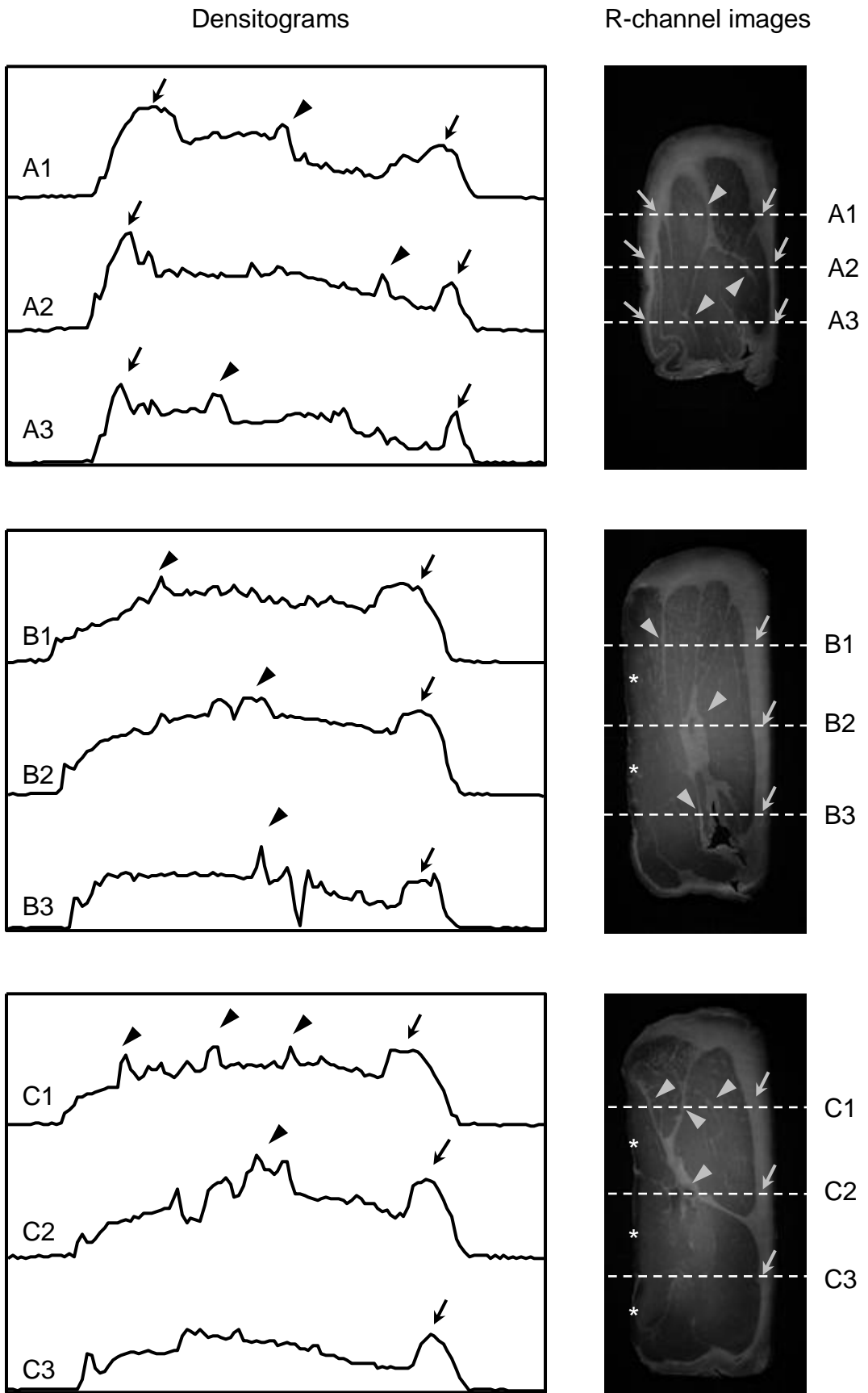


Fig. 4

Table 1. Difference between ZPP contents calculated from R values of muscle and fat in each Parma ham section.

	ZPP contents calculated from R values (no unit)		
	Section A	Section B	Section C
Lean meat	52.4 ± 16.8 ^{b·y} (32.1)	61.3 ± 20.4 ^{a·x} (33.3)	51.3 ± 19.8 ^z (38.6)
Fat	56.6 ± 23.4 ^{a·y} (41.3)	59.7 ± 27.8 ^{b·x} (46.6)	55.3 ± 26.0 ^z (47.0)

Upper values are means ± SD, lower values are CV[%] (coefficient of variation).

^{ab} Means within the same column bearing the different superscript are significantly different ($P < 0.01$).

^{xyz} Means within the same row bearing the different superscripts are significantly different ($P < 0.01$).

Table 2. ZPP contents calculated from R values in each part of Parma ham.

	ZPP contents calculated from R values (no unit)		
	Section A	Section B	Section C
Muscle (lean meat)			
Semimembranosus	61.7 ± 6.9 ^{b·y} (11.2)	54.8 ± 19.7 ^{d·y} (17.7)	45.6 ± 16.5 ^{d·z} (36.2)
Semitendinosus	70.1 ± 6.6 ^{a·y} (9.4)	78.0 ± 9.8 ^{b·x} (12.8)	50.3 ± 14.1 ^{c·z} (28.0)
Biceps Femoris	33.4 ± 7.6 ^{e·y} (22.8)	70.3 ± 15.6 ^{c·x} (22.2)	69.8 ± 10.4 ^{b·x} (14.9)
Gracilis	61.2 ± 15.9 ^{b·x} (26.0)	33.5 ± 12.5 ^{e·y} (37.3)	16.5 ± 6.4 ^{f·z} (38.8)
Quadriceps Femoris	-	-	44.2 ± 19.5 ^d (44.1)
Pectineus	-	-	36.5 ± 8.8 ^e (24.1)
Triceps Surae	50.3 ± 12.9 ^d (25.6)	-	-
Fat			
Subcutaneous fat	56.1 ± 24.2 ^{c·x} (43.1)	53.6 ± 25.2 ^{d·y} (47.0)	49.4 ± 22.6 ^{c·z} (45.7)
Intermuscular fat	61.1 ± 15.3 ^{b·z} (25.0)	82.7 ± 25.0 ^{a·y} (30.2)	87.6 ± 17.9 ^{a·x} (20.4)

Upper values are means ± SD, lower values are CV[%] (coefficient of variation).

^{abcdef} Means within the same column bearing the different superscripts are significantly different ($P < 0.01$).

^{xyz} Means within the same row bearing the different superscripts are significantly different ($P < 0.01$).