Scientific paper

Determination of Selected Protoporphyrins in Parma Ham With use of 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin as a Surrogate Standard in the Recovery Study

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Received: 25-03-2014

Abstract

A high-performance liquid chromatographic method for the determination of hemin, protoporphyrin IX (PPIX), and zinc(II)protoporphyrin IX (Zn(II)PPIX) in Parma ham was developed. The detection was done by means of a universal DAD-detector, whereby quantification of the three naturally occurring protoporphyrins was carried out at λ = 414 nm, i.e., very close to the respective maxima of their Soret bands. The extraction thereof from the meat matrix was done by a mixture of acetone and chloroacetic acid (100 mL + 0.2 g). Usage of 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin (THPP) as a surrogate standard and its detection fixed at λ = 444 nm, allowed to obtain accurate (ca. 96%) recovery results. Established concentrations of hemin, Zn(II)PPIX, and PPIX in the Parma ham samples were 15.97, 19.96 and 1.52 μg g^{-1}, respectively.

Keywords: Parma ham; hemin; protoporphyrin IX; zinc(II)protoporphyrin IX; 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin; high-performance liquid chromatography

1. Introduction

For the manufacturing of meat products, sodium nitrite (E250) and/or potassium nitrate (E252) are conventionally used. Technologically, these chemical additives contain many advantages, like the antimicrobial and antioxidative properties whereby a prolonged shelf life can be obtained. In addition, nitrite is especially added to the meat for its color developing properties.[1] However, the use of nitrite is controversial because of its toxicity (lethal oral dose: 33 mg kg^{-1} body weight) and the formation of carcinogenic *N*-nitrosamines.[2–4] Therefore, the addition of these nitrite salts to meat products is legally restricted to 150 mg kg^{-1} meat (expressed as $NaNO_2$ per kg meat).[5] Moreover, the consumers openly express a desire to avoid chemical food additives (E-numbers) in their daily diet.

Traditionally, nitrosation of the meat pigment, i.e., myoglobin, by an addition of nitrite is considered the only way to provide the processed meat products their attractive red color. Nevertheless, some exceptions can be found. For instance, the red color of a traditional Italian dry ham, i.e., Parma ham, is not achieved by the formation of nitrosyl myoglobin. Instead, zinc(II)protoporphyrin IX (Zn(II)PPIX) was identified as the red coloring pigment.[6–9] Although the mechanism of the Zn(II)PPIX action in Parma ham is not yet sufficiently understood, it might seem an interesting idea to eliminate nitrites and nitrates in the dry fermented sausage products by Zn(II)ZPPIX, as a naturally occurring coloring pigment. However, before this can be applied to other meat pro-

ducts, it would be most advisable to elucidate the color formation pathway in the existing products like, e.g., Parma ham. To do that, firstly hemin, protoporphyrin IX (PPIX), and Zn(II)PPIX (see Fig. 1), which are biochemically interrelated, must be stoichiometrically quantifiable in Parma ham. Therefore, it is important to rely on an analytical method for the determination and quantification of the most important native protoporphyrins present in the meat and meat products.

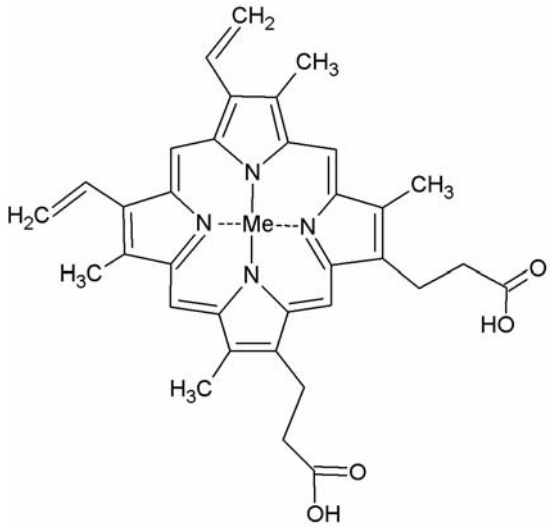

Fig. 1. The chemical structure of naturally occurring protoporphyrins in meat: hemin (Me = Fe^{2+}), PPIX (Me = /), and Zn(II)PPIX (Me = Zn^{2+}).

So far, determination of protoporphyrins in biological tissues has found interest mostly for clinical applications, whereby conventionally, PPIX and Zn(II)PPIX are quantified in the blood samples by means of spectrofluorimetry.[10] However, this method suffers from the lack of selectivity, since the fluorescence spectra of both compounds overlap and as a consequence, reliable quantification is difficult to achieve. Therefore, the results are preferentially expressed as fluorescence intensity, instead of an absolute quantification of individual porphyrins.[11,12] Introduction of the separation techniques such as high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) could circumvent the issues of selectivity. Although with use of TLC a simple and easy baseline separation method can be developed, quantification of the light sensitive compounds such, as Zn(II)PPIX becomes difficult, due to its degradation in the daylight.[13] Therefore, most current applications are based on HPLC separations, commonly followed by the fluorescence detection for the determination of PPIX and Zn(II)PPIX[12]. However, hemin shows relatively weaker fluorescence than the remaining two protoporphyrins, and its detection is mainly carried out via UV-absorption (ca. $\lambda = 400$ nm).[13] In fact, simultaneous determination of hemin, PPIX and Zn(II)PPIX in Parma ham was reported by Wakamatsu et al.,[16] whereby the necessity of using two detectors, i.e. the fluorescence and the UV-detector, was imposed. Nevertheless, these tetrapyrolic compounds are generally intensely colored, showing high absorption yields throughout the whole UV and visible range of the spectrum and thus, detection by UV-detector of these components is feasible, even with the amounts of less than one microgram.[17,18]

Despite the existence of many HPLC methods for the determination of these target analytes, so far no or little attention has been paid to applicability of the extraction procedure to the different kinds of matrices. In many cases concerning the analysis of meat and meat products, the recovery study, as part of the validation procedure, is neglected. The main reason is the lack of a representative matrix reference material, or an availability of blank samples from which the native target analytes are absent.[19]

In this study, a novel overall approach to the extraction of native hemin, PPIX, and Zn(II)PPIX from Parma ham, followed by the simultaneous HPLC-DAD quantification of these three compounds is presented and validated. To this effect, a surrogate standard, i.e., 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin (THPP) is used for the recovery study.

2. Experimental

2. 1. Reagents and Materials

Standard samples of hemin, PPIX and Zn(II)PPIX of the analytical purity grade (≤97%) were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). For the recovery purpose, THPP was used as the surrogate standard. The latter compound was synthesized and purified in-house and belongs to the group of metal-free porphyrins[20] (see Fig. 2). For analytical purposes, the standard samples of hemin, PPIX, and (Zn(II)PPIX) were dissolved in methanol of HPLC purity (Sigma-Aldrich). The employed concentrations for PPIX and Zn(II)PPIX were 0.01 mg mL^{-1}, and that of hemin was 0.05 mg mL^{-1}. The surrogate

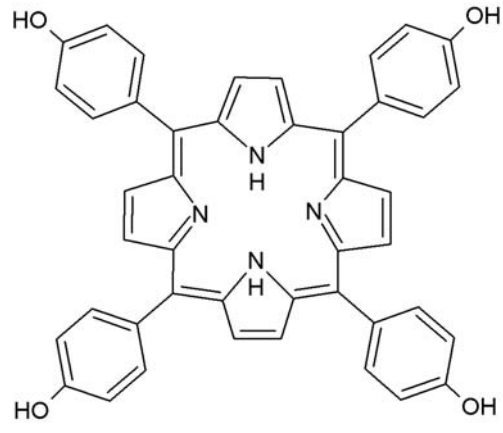

Fig. 2. The chemical structure of the surrogate standard 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin (THPP).

standard, THPP, was dissolved in acetone of HPLC purity at the concentration of 0.10 mg mL^{-1}.

For quantification of hemin, PPIX, and Zn(II)PPIX in Parma ham, the high quality Parma ham originating from a local supermarket was used. For the extraction of protoporphyrins from the meat samples, an extraction mixture composed of 100 mL acetone + 0.2 g chloroacetic acid ($C_2H_3ClO_2$) (both solvents manufactured by Sigma Aldrich) was prepared.

2. 2. High-performance Liquid Chromatography with Diode Array Detection (HPLC/DAD)

The high-performance liquid chromatographic analysis was carried out using a P580A LPG model liquid chromatograph, equipped with a Gina 50 model autosampler and a UVD340V DAD model detector (Gynkotek=Dionex, Germering, Germany). The analyses were carried out in the isocratic mode, using a Pursuit 5 C18 (5 m particle size) column (250 mm 4.6 mm i.d.; Varian; cat. no. A3000250C046). As mobile phase, a liquid mixture A + B, 9:1 (*v*/*v*) was employed, where. A: methanol (MeOH) + dichloromethane (DCM), 9:1 (*v*/*v*); B: water (H_2O) + glacial acetic acid (CH_3COOH), 97:3 (*v*/*v*). MeOH and DCM were of HPLC purity (Merck). Glacial acetic acid was of analytical purity (POCh, Gliwice, Poland) and water used in the experiment was de-ionized and double distilled in our laboratory by means of the Elix Advantage model Millipore system (manufactured in Molsheim, France). The mobile phase flow rate was 0.8 mL min^{-1}.

Calibration curves for hemin, PPIX, and Zn(II)PPIX were developed in the employed HPLC system from the chromatograms registered at λ = 414 nm (close to the maxima of the Soret bands for all three protoporphyrins), whereas the calibration curve for THPP was registered at λ = 444 nm. The retention times (t_R) for hemin, PPIX, Zn(II)PPIX, and the surrogate standard THPP were 2.6, 8.2, 5.5, and 2.8 min, respectively.

2. 3. Preparation of Extract from Parma Ham for Chromatographic Analysis

An extract of Parma ham for the chromatographic analysis was prepared according the method of Wakamatsu,[16] with some modifications. In short, 10-g Parma ham sample was first grinded with use of a cuisine hand blender (Kenwood, Havant, UK). This process was carried out in a glass vessel tightly wrapped with an aluminium foil, in order to prevent the daylight from affecting the protoporphyrins contained in the meat sample. Next, the extraction mixture (100 mL acetone +0.2 g chloroacetic acid ($C_2H_3ClO_2$)) was added to the grinded portion of meat and homogenized for 10 minutes with use of a blender in the glass vessel put on ice. Combination of the ice-cold homogenization and an addition of chloroacetic acid to the extraction mixture, promoted a relatively easy separation of fat from the Parma ham sample.

To remove solid particles, e.g., fat and proteins, from the homogenized Parma ham sample, extract separated by sedimentation from the solid matter was first filtered through a paper filter, and then through an Anotop 25 Plus syringe filter (0.02 μm, cat. no. 12085; Whatman, Maidstone, Kent, UK). To the filtrate, several drops of aqueous ammonia were added to obtain neutral pH value (pH 7). The double filtrated extract was then analyzed for the contents of hemin, PPIX, and Zn(II)PPIX by means of HPLC/DAD.

2. 4. Recovery Study

Recovery of the three porphyrins of interest, i.e., hemin, PPIX, and Zn(II)PPIX, was assessed with use of THPP as a surrogate standard. To this effect, the Parma ham sample was spiked prior to the extraction with the 1-, 2-, and 3-mL aliquots of the surrogate standard solution. Then the Parma ham was extracted and the further sample preparation procedure followed the method described in the preceding section. Each Parma ham spiking step was performed in triplicate.

2. 5. Determination of Hemin, PPIX, Zn(II)PPIX, and THPP by HPLC

For the calibration curve purpose, the peak heights for hemin, PPIX, and Zn(II)PPIX were employed at the wavelength (λ) of 414 nm, and that for THPP at the wavelength of 444 nm. This difference in the employed wavelengths was necessary, due to a small difference between the retention times of hemin (t_R = 2.6 min) and THPP (t_R = 2.8 min), and with much higher absorbance of the surrogate standard than hemin.

The calibration curves for all four porphyrins were obtained for the aliquots ranging from 0.050 to 0.250 μg mL^{-1} porphyrin (in the intervals of 0.050 μg mL^{-1}). The chromatographic peak heights were plotted against the microgram amounts of a given porphyrin injected on to the column. For each individual aliquot, six repetitions were performed (n = 6). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the formulas LOD = 3.3 × SD/a, and LOQ = 10 × SD/a, where SD is standard deviation of the peak height (n = 6) taken as a measure of noise, and a is the slope of the corresponding calibration curve (y = ax + b).

3. Results and Discussion

3. 1. Method Performance

A possibility to accurately quantify hemin, PPIX, and Zn(II)PPIX in the meat products, i.e., Parma ham, by means of the external standard calibration curves was con-

sidered. On the one hand, the use of a certified reference material (CRM) for studying the accuracy of a given analytical method is strongly recommended, yet on the other hand, the availability of the target matrices and analytes is very limited. In the case of the meat products, these CRMs are simply not available. Alternatively, the accuracy can be estimated by spiking an analyte-free matrix.[21] However, due to the natural origin of protoporphyrins in the meat tissue, representative samples are not available. Therefore, the selection of a proper surrogate analyte proved a crucial method development step. As hemin, PPIX, and Zn(II)PPIX belong to the group of porphyrins, it was decided that a metal-free porphyrin, not reported to be present in meat products and with a more intense Soret bands than the biogenic metal porphyrins, would be preferable for the quantification purpose. Different porphyrins were tested (data not shown). In comparison to the other porphyrins, i.e., 5,10,15,20-tetra(3-hydroxyphenyl)-porphyrin, the retention time of THPP was not overlapping with hemin. In addition, for quantification purposes THPP has the advantage to lack double bounds, being chemically more stable and less sensitive for light than the protoporphyrins naturally present in meat. Although THPP, used in this study, was synthesized and purified in-house, it is also commercially available and thus easily available as internal standard for the quantification of porphyrins in meat products. Because of all these advantages, THPP is selected as the most appropriate surrogate standard.

The intensity of the absorption bands for the surrogate standard was much higher than those of the target meat porphyrins. Since the retention times for hemin (2.6 min) and THPP (2.8 min) were too close to each other, selectivity was obtained by using different analytical wavelengths. For the detection and quantification of hemin, PPIX, and Zn(II)PPIX, the wavelength of $\lambda = 414$ nm was selected, while another wavelength ($\lambda = 444$ nm) for the detection and quantification of THPP was chosen. As an illustration, a comparison of the absorption spectra for hemin and THPP is given in Fig. 3.

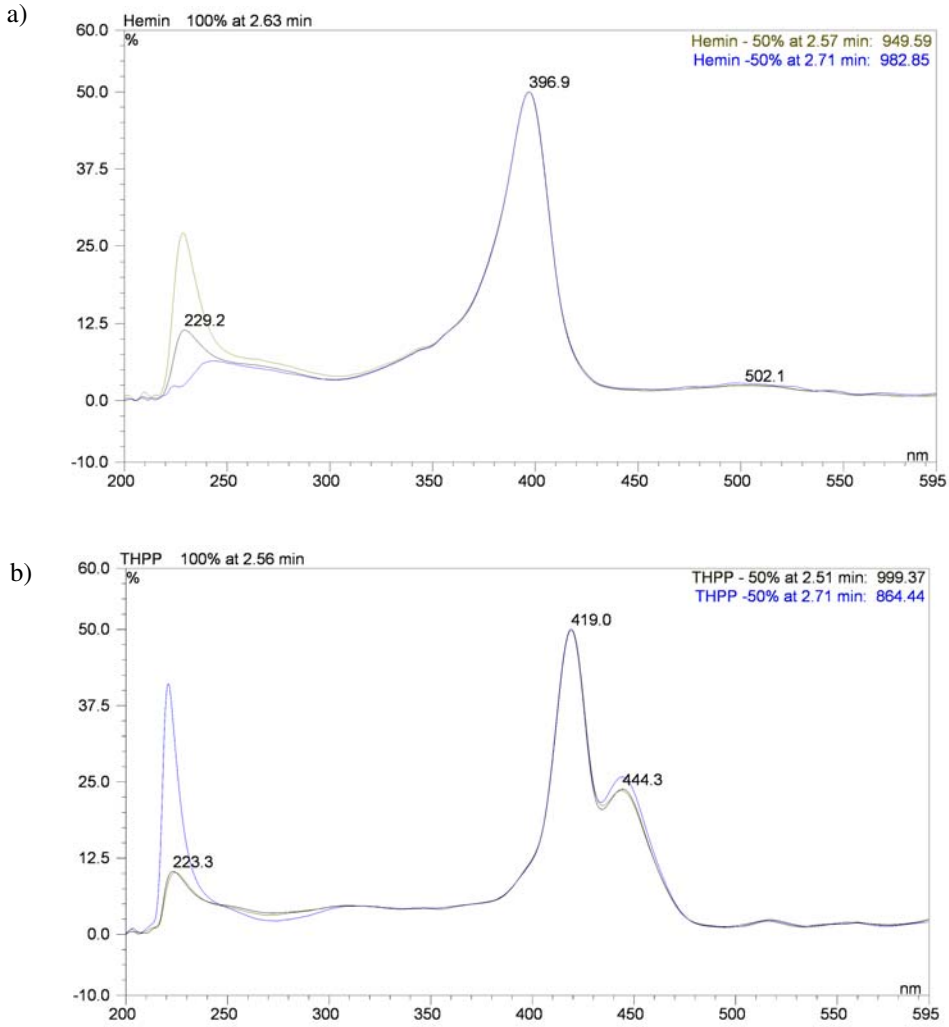

Fig. 3. The UV/Vis spectra for (a) hemin and (b) 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin (THPP) (as recorded with use of the diode array detector of the chromatograph for the two slopes of the respective chromatographic peaks).

Additionally, in Fig. 4, three chromatograms are presented. In Fig. 4a, the chromatogram registered at λ = 444 nm and valid for the Parma ham extract spiked with the surrogate standard THPP is shown. In Fig. 4b, the chromatogram registered at λ = 414 nm is presented, valid for the separated mixture of the hemin, PPIX, and Zn(II)PPIX standards, used for the preparation of the respective calibration curves. In Fig. 4c, the chromatogram (also registered at λ = 414 nm) is given, which is valid for the Parma ham extract.

The recovery results performed on the Parma ham samples spiked with the three different aliquots of THPP as a surrogate standard are summarized in Table 1.

From these results, it comes out that the recovery of the surrogate standard is practically total (ca. 96%).

Table 1 The recovery (T %) and the repeatability (r) expressed as RSD (%) of THPP from 10-g Parma ham sample spiked with three different aliquots of the surrogate standard (n = 3)

Spiked amount (μg g^{-1})	Recovery (μg g^{1})	T (%)	RSD (%)
10.0	9.42	94.2	12.8
20.0	19.24	96.2	10.7
30.0	29.31	97.7	7.8

As the chemical properties of this surrogate standard are similar to those of the analytes of interest,[20] it can be anticipated that very similar recoveries are valid for the three target porphyrins investigated in this study also (i.e., for hemin, PPIX, and Zn(II)PPIX). To this effect,

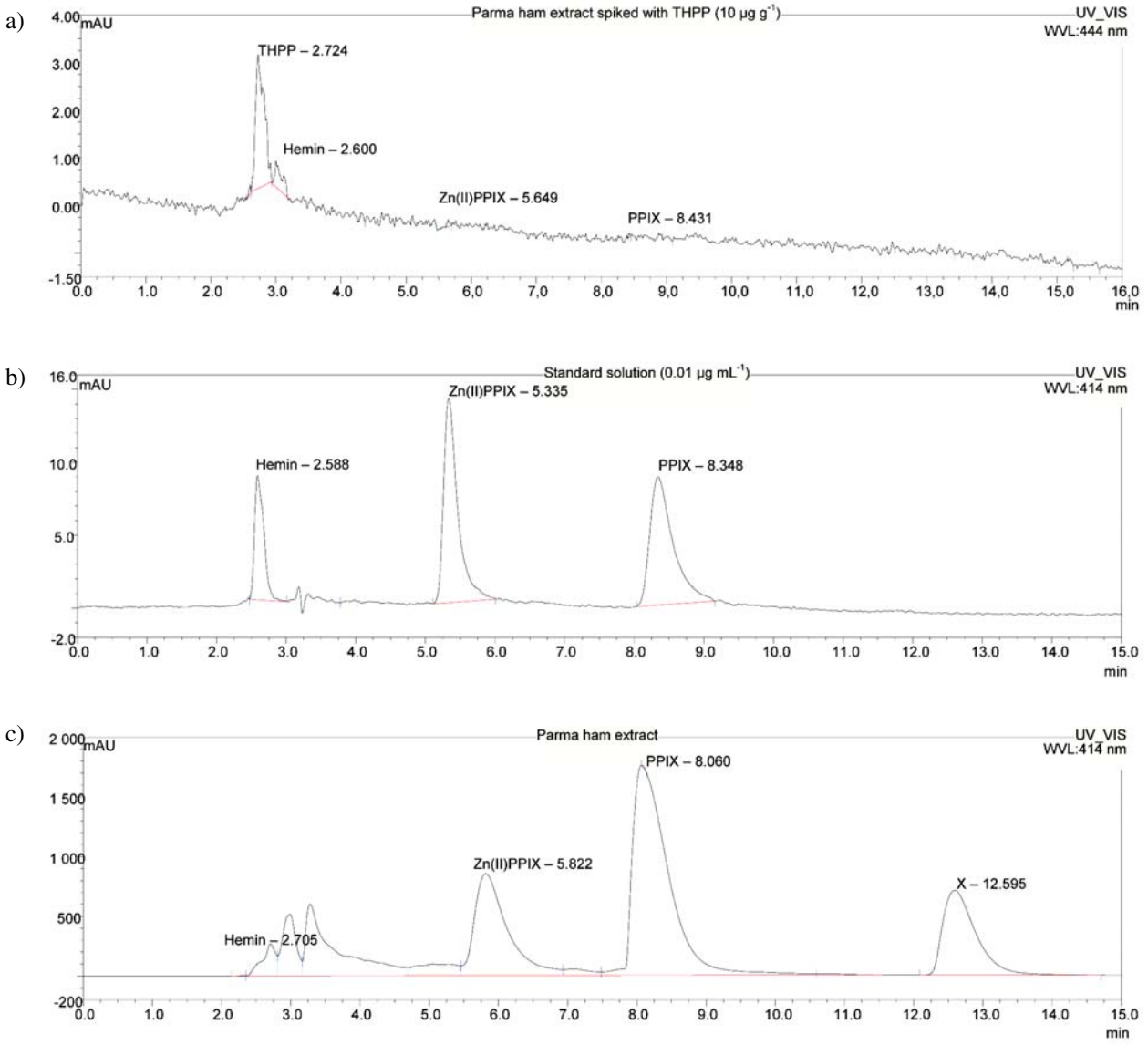

Fig. 4. (a) Chromatogram of the Parma ham extract spiked with THPP surrogate standard (10 μg g^{-1}) and registered at λ = 444 nm; (b) chromatogram of the hemin, PPIX, and Zn(II)PPIX standards (0.01 μg mL^{-1}), registered at λ = 414 nm; (c) chromatogram of the Parma ham extract without the addition of the surrogate standard, registered at λ = 414 nm.

Table 2 Calibration curves obtained from HPLC for hemin, PPIX, Zn(II)PPIX, and THPP, and the respective LOD and LOQ values (n = 6)

Compound	Calibration curve	Correlation coefficient, *r*	Standard deviation, *SD*	LOD (μg g^{-1})	LOQ (μg g^{-1})
Hemin	y = 0.1235x – 2.8672	0.9966	0.517	0.14	0.42
PPIX	y = 0.1223x – 3.5765	0.9921	0.856	0.23	0.70
Zn(II)PPIX	y = 0.1785x –2.7965	0.9654	2.399	0.44	1.34
THPP	y = 0.3497x – 0.0321	0.9965	4.361	0.41	1.25

we decided that the calibration curves obtained for these target porphyrins can further be used for quantification thereof in the real Parma ham (or the other meat) samples. The respective calibration curves for hemin, PPIX, and Zn(II)PPIX, and also for THPP, are given in Table 2. In the same table, the LOD and LOQ values for these four porphyrins are also given. Based on quantitative results summarized in Tables 1 and 2, proper quantification of the contents of hemin, PPIX, and Zn(II)PPIX was ultimately performed.

3. 2. Analysis of Parma Ham

The developed method was used for the simultaneous determination and quantification of hemin, PPIX, and Zn(II)PPIX in the Parma ham samples. As it can be seen in Table 3, the magnitude orders for Zn(II)PPIX and hemin are the same, while that for PPIX is by one magnitude order lower. In quantitative terms, the highest amount was found for Zn(II)PPIX (19.96 μg g^{-1} Parma ham), the medium amount for hemin (15.97 μg g^{-1} Parma ham), and the lowest amount for PPIX (1.52 μg g^{-1} Parma ham). Since in most studies the fluorescence intensities were reported instead of an exact quantification of individual protoporphyrins, little data is available regarding protoporphyrin concentration in the meat products such as Parma ham. Nevertheless, Wakamatsu et al.[16] were also able to extract and separate these target analytes by means of HPLC. Although in their study, another extraction solvent, namely ethyl acetate-acetic acid (4:1, v/v), was used, concentrations of the porphyrins of interest were within the same magnitude range, as established in this study. Moreover, due to the introduction of chloroacetic acid to facilitate the removal of fat from the sample matrix, our extraction method seems most promising for the analysis of protoporphyrins in the more fatty meat products such as dry fermented sausages.

Table 3 Contents of hemin, PPIX, and Zn(II)PPIX in Parma ham, as established from the chromatographic peak heights by means of HPLC (n = 6)

Compound	Amount found (μg per 1 g ham)	Precision (RSD, %)
Hemin	15.97	9.23
PPIX	1.52	11.87
Zn(II)PPIX	19.96	8.19

4. Conclusions

A simple HPLC-based analytical method for the simultaneous determination of three protoporphyrins, i.e., hemin, protoporphyrin IX, and Zn-protoporphyrin IX, was developed. The detection of all porphyrins of interest was easily done by means of a universal DAD-detector, whereby selectivity and sensitivity can be achieved by simply selecting the appropriate wavelengths for the detection and quantification purposes of the different compounds. Moreover, the accuracy of the extraction method was established by use of 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin as a surrogate standard in the recovery study. In this way, a reliable and accurate quantification method was developed for the determination of protoporphyrins in meat products. Its good performance could be confirmed by the analysis of a Parma ham sample, wherein the measured concentrations of the three protoporphyrins of interest were in the same magnitude range as that already reported in the literature.

5. Acknowledgements

This work was performed in the framework of the $MeCagrO_2$ project “Safe products, sustainable processes and employment increased attractiveness for companies from the 2 Seas agro-food Area”. Note: “The document reflects the author’s views. The interreg IVA 2 Seas Programme Authorities are not liable for any use that may be made of the information contained therein.”

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Povzetek

Razvili smo analizno metodo za določanje hemina, protoporfirina IX (PPIX) in cinkovega(II)protoporfirina IX (Zn(II)PPIX) v Parma šunki. Metoda temelji na tekočinski kromatografiji visoke ločljivosti. Za detekcijo smo uporabili univerzalni DAD detektor in kvantifikacijo teh treh naravno prisotnih protoporfirinov izvedli pri $\lambda = 414$ nm, kar je zelo blizu maksimumov njihovih Soretovih pasov. Ekstrakcijo analitov iz mesnega matriksa smo izvedli z acetonom in kloroocetno kislino (100 mL + 0,2 g). Uporaba 5,10,15,20-tetra (4-hidroksifenil) porfirina kot internega standarda in njegova detekcija pri $\lambda = 444$ nm je omogočila točne rezultate izkoristkov (~96 %). V vzorcih Parma šunke smo določili 15,97 μg g^{-1} hemina, 19,96 μg g^{-1} Zn(II)PPIX in 1,52 μg g^{-1} PPIX.