



Analytical Methods

Identification of mechanically separated meat using multivariate analysis of 43 trace elements detected by inductively coupled mass spectrometry: A validated approach

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This work is dedicated to the loving memory of our friend and colleague Giuliana Marchesani.

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ABSTRACT

The European Food Safety Authority asked for novel approaches for identifying mechanically separated meat (MSM) in meat products, due to food safety concern.

In this study, a novel approach based on multivariate analysis of 43 trace elements in meat products is described. Overall, 27 trace elements and 16 rare earth elements were determined by using ICP-MS after sample mineralization of 100 meat samples, composed of different percentages of MSM, obtained at low and high pressure, and without MSM.

After development and optimization, the multivariate approach was validated by analyzing and then classifying 10 "blind" meat samples, obtaining method accuracy equal to 90%. Thus, the applicability of this new analytical approach was demonstrated. The method represents a significant improvement for this type of determination, especially when MSM is obtained at low pressure, since this product is characterized by chemical characteristics very similar to fresh meat.

1. Introduction

Although the large consumption of meat products was discouraged by the International Agency for Research on Cancer (IARC) and Food and Agriculture Organization (FAO), due to the proved health effects on humans, the production and commercialization of meats is increasing, especially in developing countries (IARC, 2015). Among different factors linked to possible adverse effects caused by meat consumption (Iammarino, 2020; Iammarino et al., 2017), in the last years the attention of scientific community focused to a special type of meat product, mechanically separated meat (MSM). According to EC Regulation 853/2004, MSM is referred to "the product obtained by removing meat from flesh-bearing bones after boning or from poultry carcasses, using mechanical means resulting in the loss or modification of the muscle fiber structure" (European Commission, 2004).

MSM is usually obtained from poultry and swine carcasses, and the deboning processing may be carried out by using high pressure ($P \geq 10^4$ kPa) or low pressure ($P < 10^4$ kPa) technology (European Food Safety

Authority, 2013). The correct identification and classification of both types of meat is important for evaluating the overall quality (nutritional, organoleptic, etc.) of the product. However, the food safety aspects are more important, since the first type of meat is characterized by higher degree of muscle fiber destruction, which makes it more perishable than traditional minced meat (Viuda-Martos et al., 2012). For this reason, the presence and the percentage of MSM have to be declared on the product label.

In this regard, the BIOHAZ Panel of the European Food Safety Authority (EFSA) recommended the Organisms in charge of food inspections, such as veterinary services, reference laboratories, etc., for the development of new approaches useful for the identification of MSM (EFSA, 2013). This recommendation was due to the available tools for this purpose (i.e. rheological/textural parameters, protein, ash, cholesterol, iron, etc.), judged as not fully reliable. Among all tested parameters, only calcium content is significantly correlated with the presence of MSM in meat products, and a concentration of this element higher than 1000 mg kg^{-1} is attributed to the presence of MSM (European

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Commission, 2005). These high levels of calcium, especially in MSM obtained at high pressure, are due to this processing which leads to the fragmentation of bone parts and periosteum (bone skin) (Branscheid et al., 2009; European Commission, 2001; Tremlová et al., 2006). However, the percentage of bone micro-fragments in meat products containing MSM is highly variable, due to the different percentages of MSM added in the formulation, and to the bone fragmentation phenomenon, which is obviously unpredictable. Moreover, very high concentrations of calcium may be present also in non-MSM products containing permitted food additives, such as calcium ascorbate (E302) or calcium citrate (E333) and/or particular ingredients rich in calcium (i.e. cheese, concentrated plant extract, etc.) (Iammarino et al., 2020). As a consequence of these considerations, “false-negative” or “false-positive” responses could be registered during official control.

Some alternative analytical approaches for identifying MSM have been proposed during the last years. These methods are based on total reflection X-ray fluorescence (Dalipi et al., 2018), sample irradiation coupled to electronic spin resonance (Tomaiuolo et al., 2019), multivariate approaches (Iammarino et al., 2020), ion chromatography (Iammarino et al., 2021), X-ray micro computed tomography (Pospiech et al., 2019), ultrasonic method (Wieja et al., 2021) and histological techniques (Guelmamene et al., 2018). Some interesting approaches for evaluating MSM properties were also proposed by using Raman and Near Infrared (NIR) spectroscopy (Andersen et al., 2015; Wubshet et al., 2019). Looking at the available literature, the authors verified that methods able to identify MSM in different types of meat products are lacking. In particular, most proposed approaches were only tested on particular types of meat (i.e., minced meat, sausage, etc.). Moreover, few references to low pressure and high pressure MSM are available and the validation procedures are lacking. This means that these procedures are useful as “screening” techniques, but a confirmatory method is still not available.

The main objective of this study was the development of a confirmatory method for the identification of MSM in meat products, by using inductively coupled mass spectrometry (ICP-MS) as the analytical technique, and multivariate analysis for data elaboration. More specifically, ICP-MS simultaneously provided 43 analytical data which were elaborated by means of chemometrics, properly. A novel approach for method validation was also reported.

2. Materials and methods

2.1. Sampling

Overall, 100 samples, composed of different types of fresh meats, meat preparations and meat products were collected from local stores during the years 2017–2020, and then analyzed within the activities of the “MPSQA” research project, carried out at Chemistry Department of Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (Foggia, Italy). The storage temperature has no significant effect on the amount of trace elements in the products since there is no scientific evidence. However, in order to avoid any chemical-physical change of product characteristics, once arrived in laboratory the samples were stored at $-18\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ until analysis.

The authors developed the method optimization with special emphasis on its applicability to a wide range of meat products. An important result to achieve was represented by the capability of this innovative approach to identify the MSM presence in meat products regardless of species (chicken/swine), cooking processes (baking, thermal cooking, etc.), and other characteristics influencing products composition, notably. The samples were collected in order to analyze an equal number of samples (50) composed of mechanically separated meat (MSM) and not mechanically separated meat (NO-MSM). Moreover, among MSM samples, a further subdivision was possible between products containing mechanically separated meat obtained at low pressure (LP-MSM) and high pressure (HP-MSM). This classification was

obtained referring to the product label, which also indicated the type and percentage of added MSM.

The following samples were collected and analyzed: 50 MSM samples (30 HP-MSM: 19 chicken/turkey wurstel, 9 chicken/swine wurstel, 2 chicken wurstel; 20 LP-MSM: 10 chicken breaded meat, 10 chicken/turkey breaded meat); 50 NO-MSM samples (8 chicken fresh meat, 9 swine fresh meat, 4 swine sausage, 7 chicken/turkey wurstel, 11 swine wurstel, 2 cooked ham, 3 baked chicken breast, 3 baked turkey breast, 3 swine hamburger). The percentages of MSM in these samples, as declared on the product labels, were in the range 30–94 % and 20–47 % for HP-MSM and LP-MSM respectively.

2.2. Chemicals, working standard solutions and materials

68 % (v/v) HNO_3 , 30 % (v/v) H_2O_2 and ultrapure water were purchased from Romil Ltd. (Cambridge, UK); single element standard solutions (Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sn, Sr, Tl, V and Zn, 1000 mg L^{-1}) were supplied by CPAchem Ltd. (Bogomilovo, Bulgaria); single element standard solution of U was supplied by Inorganic Ventures (Christiansburg, Virginia, USA); multi-element standard solutions (100 mg L^{-1}) containing Rare Earth Elements (Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Tb, Tm, Y and Yb) and Th were supplied by Inorganic Ventures (Christiansburg, Virginia, USA). Ultrapure argon (99.9999 % purity) and ultrapure helium (99.9999 % purity) were purchased from SAPIO s.r.l. (Milan, Italy). For quality assurance purposes, the following standard reference materials were used: SRM 1577c bovine liver supplied by National Institute of Standards and Technology (NIST), ERM-BB184 bovine muscle, BCR-186 pig kidney and BCR-185R bovine liver supplied by the European Commission's Joint Research Centre (JRC).

2.3. Analytical procedure

This study was carried out using the principles of two reference methods: EN 13805:2014 “Determination of trace elements - Digestion under pressure” and EN 15763:2009 “Determination of arsenic, cadmium, mercury and lead in food” for the determination of all elements, in a modified and combined version. About 100 g of each sample were homogenized by a cutter homogenizer (Grindomix GM 200, Retsch GmbH, Haan, Germany). Mineralization step was carried out by using an Ethos-One Microwave Reaction System (Milestone s.r.l. Sorisole, Bergamo, Italy): $1.00\text{ g} \pm 0.0001\text{ g}$ of homogenized sample was weighed into a Teflon vessel using an analytical balance (Mettler Toledo s.p.a., Novate Milanese, Milan, Italy); 6 mL of 68 % (v/v) HNO_3 and 2 mL of 30 % (v/v) H_2O_2 were added and the vessels were placed into microwave reaction system. The complete digestion was obtained through the following program: up to $120\text{ }^{\circ}\text{C}$ in 15 min and constant for 10 min; up to $200\text{ }^{\circ}\text{C}$ in 15 min and constant for 20 min; cooling stage (30 min) to reach room temperature. After digestion, the solution was transferred into polypropylene disposable tubes and diluted to 50 mL with ultrapure water for subsequent instrumental analysis.

An inductively coupled plasma quadrupole mass spectrometer NexIon 2000 (PerkinElmer Inc., Massachusetts, USA) equipped with concentric nebulizer (Meinhard Associates, Golden, USA), demountable quartz torch with 2.0 mm i.d. quartz injector tube and quadrupole ion deflector, was used for trace element quantification. The following operational parameters were set: nebulizer gas (Ar) flow rate: 1.01 L min^{-1} ; plasma gas (Ar) flow rate: 15 L min^{-1} ; auxiliary gas flow rate: 1.2 L min^{-1} ; radio frequency power: 1600 Watt.

2.3.1. Trace elements determination

The following isotopes were detected: ^{107}Ag , ^{27}Al , ^{75}As , ^{138}Ba , ^9Be , ^{44}Ca , ^{111}Cd , ^{59}Co , ^{52}Cr , ^{63}Cu , ^{56}Fe , ^{202}Hg , ^7Li , ^{24}Mg , ^{55}Mn , ^{98}Mo , ^{23}Na , ^{60}Ni , ^{120}Sb , ^{82}Se , ^{118}Sn , ^{88}Sr , ^{205}Tl , ^{238}U , ^{51}V and ^{66}Zn . In order to consider the intrinsic variability of lead isotope distribution and to improve the signal sensitivity, the sum of ^{206}Pb , ^{207}Pb and ^{208}Pb was

counted. Rhodium and bismuth (both at 200 ng mL⁻¹) were used as internal standards added to standard and sample solutions by a multi-channel valve. Collision cell technology was used to minimize isobaric interferences, employing helium gas (100 %, high purity) at 4.8 mL min⁻¹ for the determination of Al, Cr and Fe and at 3.7 mL min⁻¹ for the determination of As, Co, Cu, Fe, Mn, Ni, V and Zn. The instrumental calibration was performed by standard addition procedure (5 addition levels including non-added level). A preventive and semi-quantitative analysis was carried out to define the concentration rank, for each element, in order to select the most suited calibration range. The addition levels in the mineralized solutions were: Ag, Be, Cd, Co, Hg, Sb, Sn, Tl and U (0.004 – 0.02 – 0.10 – 0.40 ng mL⁻¹); As, Li, Mo, Ni, Pb, Se and V (0.040 – 0.20 – 1.0 – 4.0 ng mL⁻¹); Ba, Cr, and Sr (0.20 – 1.0 – 5.0 – 20.0 ng mL⁻¹); Al, Cu and Mn (0.40 – 2.0 – 10.0 – 40.0 ng mL⁻¹); Fe and Zn (1.0 – 5.0 – 25.0 – 100.0 ng mL⁻¹); Ca (10.0 – 50.0 – 250.0 – 1000.0 ng mL⁻¹). A good linearity was observed in the calibration range set for each element with determination coefficient (R²) higher than 0.995. The goodness-of-fit of the data of calibration curve was checked by Mandel test. Mg and Na were determined only by semi-quantitative analysis. Limit of quantification (LOQ) values of the method, for each element, were determined by blank determination assays, as 10 times the standard deviation of 20 blank replicates (see [Supplementary Table 1](#)). The accuracy of the method was verified by using four certified reference materials (CRMs): SRM 1577c bovine liver, ERM-BB184 bovine muscle, BCR-186 pig kidney and BCR-185R bovine liver (see [Supplementary Table 2](#)). The performances of the analytical method are reported in [Supplementary Table 1](#). Recovery factors were determined by using ERM BB 184 bovine muscle reference material. For those elements not included in the list of certified parameters, spiking tests were carried out by addition of standard mono-element solution. Recovery factors ranged from 82 % (Se) to 123 % (Fe). The results were not corrected for recovery factor because they were statistically comparable to 100 %. Two replicates of each meat sample were analysed, and the trace element concentrations were evaluated as the mean of both measurements. A good repeatability (less than 10 %) was obtained for all the determinations.

2.3.2. Rare earth elements determination

For Rare Earth Elements (REE), the following isotopes were detected: ¹⁴⁰Ce, ¹⁶⁴Dy, ¹⁶⁶Er, ¹⁵³Eu, ¹⁵⁸Gd, ¹⁶⁵Ho, ¹³⁹La, ¹⁷⁵Lu, ¹⁴²Nd, ¹⁴¹Pr, ¹⁵²Sm, ¹⁵⁹Tb, ¹⁶⁹Tm, ⁸⁹Y, ¹⁷⁴Yb and ²³²Th. Gallium, rhenium and bismuth (both at 200 ng mL⁻¹) were used as internal standards, added to standard and sample solutions by a multi-channel valve. The instrumental parameters were set to minimize the formation of oxides to a maximum of 0.5 %, in order to make negligible the potential isobaric interferences. The instrumental calibration was performed by standard addition procedure, by using the common multi-element standard solution. The addition levels were: 0.004 – 0.02 – 0.1 – 0.4 ng mL⁻¹. A good linearity was observed in the calibration range set for each element with determination coefficient (R²) higher than 0.995. The goodness-of-fit of the data of calibration curve was checked by Mandel test. LOQ values of REEs, determined by blank determination assay and quantified as 10 times the standard deviation of 20 blank replicates, were equal to: 69 (Ce), 3.9 (Dy), 3.6 (Er), 1.8 (Eu), 12 (Gd), 2.2 (Ho), 32 (La), 0.63 (Lu), 44 (Nd), 8.5 (Pr), 3.3 (Sm), 2.0 (Tb), 0.63 (Tm), 26 (Y), 3.2 (Yb), and 17 (Th) (expressed as pg g⁻¹ wet weight). No specific reference material certified for the content of REE is available, so the recovery factors were measured by spiking tests, and they ranged from 91 % to 110 %. The results were not corrected for recovery factors because they were statistically comparable to 100 %. Two replicates of each meat sample were analysed, and the REE concentrations were evaluated as the mean of both measurements. A good repeatability (less than 10 %) was obtained for all determinations.

2.3.3. Statistical analysis

The mean concentration, standard deviation, median and range were

calculated for each trace element and for the three types of meat products considered (NO-MSM, LP-MSM and HP-MSM). In order to find the elements that significantly changed between groups ($p < 0.05$), comparisons between groups were carried out by one-way ANOVA using the concentration data normalized by log-transformation. Fisher's Least Significant Difference (LSD) was used for post-hoc tests, in order to assess the inter-groups differentiation. Principal component analysis (PCA) was carried out using MetaboAnalyst 5.0 web application, based on the R computing system (version "R v3.6.3" of February 2020, Xia Lab, Montreal, QC, Canada). As the variables had large differences in scaling, normalization was performed by using variance scaling method ([Chong et al., 2019](#)).

2.3.4. Multivariate data processing

According to the sampling scheme, the data matrix submitted to the multivariate data processing was constituted by 110 rows, one for each sample analyses, and 43 columns, corresponding to the chemical parameters analysed. The samples of the validation set were chosen according to the commercial availability, without any previous information about their meat category; the membership was, in fact, determined by lab analysis only after obtaining model results. It is important to point out that samples of the validation set were analysed at a later time and independently from the calibration set; this systematic difference in terms of analytical session was planned for testing the model under real and challenging conditions.

Taking into account that most of the parametric methods rely on the assumption of normal distribution of the data, the Lilliefors normality test ([Lilliefors, 1967](#)) was initially performed on all 43 variables, and EDA (Exploratory Data Analysis) plots ([Tukey, 1977](#)) were built as a complementary graphical technique, assessing the distributions of the 43 individual variables. An EDA plot combines four plots together: box and whiskers, histogram, probability density and scatter plots. After having auto-scaled column-wise the 100 calibration samples, principal component analysis (PCA) was applied to the data matrix as a display method for identifying differences and groupings among samples according to their category (HP-MSM, LP-MSM or NO-MSM) ([Jolliffe, 2002](#)). Thanks to the projection of the 10 commercial samples in the orthogonal space defined by a reduced number of selected components, an initial evaluation of the suitability of the dataset for a supervised classification strategy was performed ([Massart et al., 1998](#)). Subsequently, linear discriminant analysis (LDA) was applied with the final goal of developing a discriminant rule able to predict the membership of new samples to one of the three meat categories under study. LDA was selected as the discriminant method since linear delimiters usually provide simpler and more robust discriminant rules, when compared with the non-linear ones.

In more detail, LDA is a probabilistic classification technique that postulates a multivariate normal distribution inside every class under study and the same dispersion (i.e., the same variance-covariance matrix) in all of them. After model development and optimization, the prediction ability was evaluated by applying the mathematical function onto the 10 commercial samples. In this way, a robust validation of the proposed strategy was performed. The figures of merit used for evaluating the model goodness were the total classification rate (TR) and the category classification rate (R_c):

$$TR = \frac{\sum_c m_{cc}}{N} \quad (1)$$

$$R_c = \frac{m_{cc}}{N_c} \quad (2)$$

where N is the number of total classifications and m_{cc} is the number of objects of category c correctly assigned to category c. Lastly, in order to understand which variable contributes most to the discrimination among classes, Stepwise-Linear Discriminant Analysis (STEP-LDA) was employed as feature selection technique in order to retain the elements

with significant differences and then build a 'more parsimonious' LDA discriminant model. STEP-LDA is probably the oldest method of variable selection for classification techniques. This algorithm selects the variables producing the largest decrease of the Wilks Lambda, which is an inverse measure of the ratio between inter-categories and intra-category variances.

3. Results and discussion

3.1. Overall remarks

Chemometrics was used in this paper, extensively. In this first section of results discussion, given the high number of data available, related to micronutrients and contaminants, and the lack of similar papers in the literature, 20 more interesting elements were taken into account for nutritional and toxicological evaluations. This section does not rely with the analytical approach, but it deserves *ad-hoc* discussion, due to remarkable findings and new research perspectives. The elemental concentrations detected in 100 analysed samples are reported in [Supplementary Table 3](#). Moreover, an accurate description of collected samples is reported in [Supplementary Table 4](#). No meat product with added food additives containing calcium was found on the market. However, several samples were composed by ingredients containing high amounts of calcium, such as cheese, powdered milk, vegetable extracts, etc. For data statistical analysis, analytical results below the limit of quantification were imputed as $LOQ/\sqrt{2}$ (Hornung & Reed, 1990). A primary classification of elemental content in these samples was made looking upon their function (essential trace elements, non-essential trace elements and rare-earth elements). On the basis of the obtained results and the nutrition/toxicological significance, 20 elements (10 essential trace elements and 10 non-essential trace elements) were selected and used for statistical evaluations.

The values obtained in terms of mean, standard deviation (SD), median and concentration range, relating to 10 essential trace elements (Fe, Se, Co, Zn, Mn, Cu, Ca, Mo, Mg and Na) are presented in [Table 1](#). The ANOVA results were also reported by specifying, for each element, the p-value and the group pairs significantly different, as emerged by post-hoc test.

Calcium concentration is remarkably higher in HP-MSM samples,

with a mean value of 1019 mg kg^{-1} . In particular, 17 samples were characterized by concentration higher than 1000 mg kg^{-1} , which is the threshold of Ca content considered for identifying a MSM, as proposed by the Regulations (EC) No. 2074/2005 (European Commission, 2005). On the contrary, in LP-MSM products, no sample above 1000 mg kg^{-1} was detected (highest level: 807 mg kg^{-1}). In NO-MSM samples, Ca content was 10 times lower than HP-MSM, with a mean value of 125 mg kg^{-1} . According to these values and the indications reported by the European Commission and EFSA, Ca was then confirmed as the best quantitative indicator for the classification and confirmatory testing of MSM products. However, this parameter alone does not allow the differentiation between LP-MSM and fresh meat products (EFSA, 2013).

A similar trend may be observed for Fe content. The lower values were quantified in NO-MSM, while the concentrations were 2/2.5 times higher in LP-MSM and HP-MSM samples, respectively. This contamination may be imputable to some processing steps. In fact, some studies demonstrated that Fe content of MSM may rise until 70 % with respect to unprocessed poultry meat (Barbut, 2002). Most likely, this increase is due to the release of haemoglobin from bone marrow during the processing, when the bones are crashed (Daros et al., 2005; Froning, 1981).

An opposite tendency was verified for Mg concentration, which was significantly higher in NO-MSM if compared to HP-MSM samples. This result is interesting; in fact, the PCA loading plot ([Suppl. Fig. 1B](#)) confirmed that the variable Mg concentration is associated with NO-MSM samples. The same trend for Mg and Ca was reported in a recent study of MSM identification by suppressed cation-exchange chromatography. Actually, an inverse correlation between Ca and Mg concentration was observed (Iammarino et al., 2021). Undoubtedly, this aspect is very interesting, deserving further research for possible explanations about the higher levels of Mg found in fresh meats if compared to processed.

By observing loading plot, HP-MSM and particularly LP-MSM samples were characterized by higher content of Co, Mn and Mo. These trace elements are minor constituents of stainless steel and, although few data are available on metal release in the food industry, a slightly migration phenomenon cannot be excluded (Jellesen et al., 2006). On the contrary, no significant difference emerged for Se (p-value = 0.99) and Zn (p-value = 0.066) concentrations. The concentrations of 10 non-essential trace elements (Al, Cr, Ni, As, Sr, Cd, Hg, Tl, Pb and U) together with

Table 1

Concentrations ($\mu\text{g/g}$ wet weight) and statistical analysis of ten selected oligoelements in High Pressure (HP), Low Pressure (LP) and not mechanically separated (NO) meat samples.

		Co*	Mo	Fe	Se	Zn	Mn	Cu	Ca	Mg	Na
	p-value	1.8×10^{-6}	2.2×10^{-25}	1.7×10^{-22}	>0.05	>0.05	3.2×10^{-36}	1.1×10^{-6}	4.4×10^{-32}	2.1×10^{-7}	2.4×10^{-4}
	Post-hoc test	HP - NO LP - NO	LP - HP HP - NO LP - NO	HP - LP HP - NO LP - NO	-	-	HP - NO LP - NO	LP - HP LP - NO	HP - LP HP - NO LP - NO	LP - HP NO - HP NO - LP	HP - NO LP - NO
High Pressure MSM (n = 30)	Mean	4.03	0.0435	17.9	0.124	16.3	0.243	0.415	1019	161	6670
	SD	1.84	0.0074	3.41	0.024	2.63	0.038	0.047	250	13	2030
	Median	3.21	0.0421	17.0	0.119	16.8	0.232	0.416	1038	163	6390
	Min	1.80	0.0290	13.0	0.084	9.76	0.177	0.324	553	137	4650
	MAX	8.31	0.0634	27.2	0.182	20.6	0.372	0.534	1845	186	14,100
Low Pressure MSM (n = 20)	Mean	4.17	0.111	14.3	0.124	12.3	2.04	0.667	511	194	7630
	SD	2.54	0.039	4.69	0.021	2.83	1.02	0.153	199	28	2300
	Median	4.43	0.121	14.4	0.124	12.1	1.61	0.678	522	201	7970
	Min	0.086	0.037	6.44	0.087	8.87	0.24	0.355	169	141	3330
	MAX	7.83	0.180	24.3	0.167	18.2	3.88	0.951	807	231	10,500
NO MSM (n = 50)	Mean	1.58	0.0184	7.07	0.126	16.5	0.150	0.446	125	237	5890
	SD	1.15	0.0100	2.73	0.028	10.1	0.086	0.194	125	68.4	5260
	Median	1.39	0.0160	7.09	0.130	15.1	0.118	0.405	92.6	251	5010
	Min	0.155	0.0042	3.40	0.063	5.32	0.044	0.192	35.6	111	339
	MAX	4.91	0.0384	13.3	0.202	53.2	0.520	1.070	661	343	15,500

*Expressed as ng g^{-1} wet weight.

SD, range, mean and median values are shown in Table 2. The selection of these 10 elements was made up considering their potential toxicological interest. Among them, the maximum levels (MLs) permitted in meats were set in the Regulation (EC) No. 1881/2006 (European Commission, 2006) only for Pb and Cd. The MLs correspond to 0.10 and 0.05 mg kg⁻¹ wet weight, for Pb and Cd, respectively, in “meat (excluding offal) of bovine animals, sheep, pig and poultry”. All analysed samples were compliant with these limits with maximum concentrations equal to 0.0699 and 0.0218 mg kg⁻¹ for Pb and Cd, respectively (Table 2). The ANOVA results were also reported in Table 2 by itemizing, for each element, the p-value and the group pairs significantly different as confirmed by post-hoc test.

On the PCA evidence, the three groups of samples (NO-MSM, LP-MSM and HP-MSM) did not differ markedly. The PC1, that accounted for the 55 % of total explained variance, was particularly affected by the intra-group variability of NO-MSM samples, due to the sampling strategy. Therefore, unlike essential trace elements, inter-group differentiation for these inorganic contaminants is limited (Suppl. Fig. 1C). As, Hg and Tl were found in very low concentrations in all three groups. In fact, all risk assessment studies confirmed their low tendency to accumulate in meat matrices (EFSA, 2009, 2012; Filippini et al., 2020). Slightly higher values of Pb and Cd were detected in HP- and LP-MSM. Cadmium showed the highest significant difference ($p = 6.38E-24$) and the lowest concentration in NO-MSM samples. The same consideration is valid for U ($p = 2.36E-11$), Cr ($p = 3.66E-10$) and Ni ($p = 4.84E-9$). The concentrations of Al ($p = 7.27E-11$) were significantly different in the three groups, with the highest content in LP-MSM samples, followed by HP- and NO-MSM samples. However, these data showed the greatest variability, i.e., more than 100 % in term of coefficient of variation for HP-MSM ($CV_{HP-MSM} = 110\%$; $CV_{LP-MSM} = 63\%$). Moreover, it is not possible to define the specific sources of Al (e.g., releasing during processing and storage from Al-containing foils, containers, utensils, food additives, etc.) (EFSA, 2008), but only estimating the total content.

A content of Sr higher in MSM samples was generally expected, taking into account its mimetic activity, similar properties with Ca and tropism for bone tissues (Cabrera et al., 1999). However, it is well-known that the stable and non-radioactive isotope of Sr has low toxicity (World Health Organization, 2010).

All the analysed samples showed mean Ni content of 0.058 mg kg⁻¹. This value is in accordance with data reported in the EFSA report on the

risk assessment of Ni in food and drinking water. This document reported a mean content of Ni in meat and meat products equal to 0.105 mg kg⁻¹ (lower bound approach). The slightly higher values detected in MSM samples in this study (Table 2) are probably imputable to the use of other ingredients, i.e., cereals and other vegetables (breeding layer, spices, etc.). In general, as confirmed by EFSA, meat and meat products are not a primary route of exposure to this emerging contaminant/allergen (EFSA, 2020).

Finally, it is important to underline that, during the last years, the technology of meat recovery from carcasses has been greatly improved. In fact, mechanical meat separators have become more than primitive presses, with the introduction of high-temperature and high-pressure resistant systems, made of rubber and stainless steel (EFSA, 2013). Accordingly, the general data presented above indicated that the process method does not lead to significant release of toxic elements in the product.

3.2 Univariate analysis and limitations

Meat products composed of MSM are characterized by different physico-chemical characteristics. In fact, in a such complex process as meat separation, many variables must be considered. The commercial type of machinery as well as the setting conditions, the values of pressure applied, the temperature, the availability of chilled tanks are not available information in the present dataset. The intrinsic variability of mechanically separated meat matrices is an additional source of variability. For example, it has not escaped authors' notice that the higher concentration of Mg in NO-MSM may be attributable to evaporation processes or cuts of meat used (muscle, tissues more perfused and richer in water). In this regard, the higher level of Mg in NO-MSM products is not the only interesting aspect, worthy of researching. If evaluated by univariate approach, some other elements presented particular results which deserve future deepening. Similar trends were observed for some elements, Al, Mo, Mn and minimally for Cu that were all higher in LP-MSM samples (Suppl. Fig. 2), suggesting that with this processing method migration phenomenon may be relevant (a positive correlation of this variables is also confirmed by PCA analysis). However, the limited information of processing conditions and machineries used make these hypotheses unverifiable. Animal feeding, type, geographical origin, use of other ingredients and their correlation with elemental

Table 2

Concentrations (µg/g wet weight) and statistical analysis of ten selected contaminants in High Pressure (HP), Low Pressure (LP) and not mechanically separated (NO) meat samples.

		Al	Cr	Ni	As	Sr	Cd	Hg	Tl	Pb	U
	p-value	7.2*10 ⁻¹¹	3.6*10 ⁻¹⁰	4.8*10 ⁻⁹	2.0*10 ⁻⁵	2.2*10 ⁻⁷	6.3*10 ⁻²⁴	<0.05	4.3*10 ⁻³	8.3*10 ⁻⁶	2.3*10 ⁻¹¹
	Post-hoc test	LP - HP HP - NO LP - NO LP - NO	HP - NO LP - NO	HP - NO LP - NO	HP - NO LP - NO	HP - NO LP - NO	HP - NO LP - NO	-	HP - NO	HP - NO LP - NO	HP - NO LP - NO
High Pressure MSM (n = 30)	Mean	967	134	66.2	3.11	475	7.40	0.73	1.06	8.73	1.05
	SD	1070	79	55.2	0.95	304	2.67	1.43	0.43	13.00	0.37
	Median	619	105	45.9	2.75	320	7.12	0.39	1.04	4.01	1.03
	Min	370	40	24.2	1.65	200	1.75	0.39	0.50	2.13	0.41
	MAX	6080	306	277.0	5.46	1210	14.70	8.17	2.63	69.90	1.93
Low Pressure MSM (n = 20)	Mean	2210	205	99.7	3.29	877	9.45	0.62	0.71	11.9	1.16
	SD	1400	112	72.0	0.67	393	4.59	0.78	0.45	13.1	0.23
	Median	2320	186	74.7	3.15	1090	9.35	0.39	0.54	7.7	1.18
	Min	399	72	23.6	1.97	233	2.93	0.39	0.25	2.3	0.54
	MAX	4710	412	283	4.80	1260	19.00	3.87	1.89	55.5	1.47
NO MSM (n = 50)	Mean	522	64	37.0	2.29	382	1.48	0.57	1.08	3.75	0.49
	SD	413	66	83.9	1.88	508	3.18	0.81	1.79	2.95	0.66
	Median	425	38	20.6	1.99	106	0.62	0.39	0.38	2.74	0.22
	Min	104	5	1.7	0.48	11	0.14	0.39	0.05	1.10	0.02
	MAX	1900	270	599.0	9.34	1520	21.80	5.89	10.10	15.10	3.32

composition of meat are other parameters that require specific studies, but, at the same time, make single discriminant analysis very difficult for MSM identification. Of course, the verification of such differences by “ad-hoc” studies would be very interesting, since they could also be useful to fully understand the reason why this approach works so well.

Other small differences observed for other elements as taken alone appear to have no statistical significance, if evaluated by univariate approach. So, the power of this study lies in the application of the multivariate statistical method to minimize the effect of these variables. However, these general considerations as well as the dataset proposed can be considered as extremely useful, not only for those researchers/laboratories/stakeholders who intend to replicate the analytical method, but also for monitoring programmes and regulatory agencies.

Finally, it is not less important that the development and usage of “multi-analyte or multi-parameter methods” is in line with the main principles of green analytical chemistry and sustainable green sample preparation (Gatuszka et al., 2013).

3.3 Multivariate elaboration

Regarding the statistical procedure applied on the variables for verifying their normal distribution, Fig. 1 shows, as an example, EDA plots for the variable Ni obtained for the 2 categories of MSM (including both LP-MSM and HP-MSM) and NO-MSM samples, before and after logarithmic transform. Based on the Lilliefors normality test, run at a 5 % significance level (some examples are shown in Suppl. Fig. 3), and on the EDA plots examined for all 43 variables, the following ones were considered as significantly non-normal and, subsequently, they were logarithmic transformed prior to the subsequent data processing: Al, Cr, Fe, Ni, As, Co, Cu, Li, Ag, Sr, Ba, Tl, Pd, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Er, Tm, Th, U.

Regarding the results of PCA applied on the calibration set of 100 samples, the score plot of the two lowest-order principal components

(Fig. 2A) showed sample groupings consistent with the category membership. In particular, NO-MSM samples were placed at negative values of PC1 scores, while HP-MSM and LP-MSM samples were mostly located at positive values. Looking at the corresponding loading plot (Fig. 2B), it is possible to notice that most of the variables have a positive contribution on PC1. These variables, being located in the right area of the orthogonal space, have approximately the same loading value, so that they can be considered positively inter-correlated (e.g., Ca and Ce, Fe, Nd and La). Looking at the scores in Fig. 2A, it is possible to notice that the MSM samples and NO-MSM samples discriminate along PC1. In particular, the NO-MSM samples are characterized by more negative values on PC1, and on the contrary, MSM samples have positive values on PC1 which explains 50 % of the total variance. The corresponding loadings in Fig. 2B show that most variables are at positive values on PC1 and therefore, it can be stated that the MSM samples present higher values for all the chemical properties measured. On the other hand, a distinction between the two types of MSM seems to occur along PC2: LP-MSM at positive values and HP-MSM at negative values. Combining this observation with the information provided by the loadings, it can be concluded that HP-MSM products are characterized by higher content of Ca, Ce, Be, La, Lu and Nd, and lower content of Mn, Mo, Ho and Ag, if compared to LP-MSM products. According to the proposed data processing strategy, the 10 commercial samples were projected into the orthogonal space defined by the two lowest order components (PC1 and PC2) in order to evaluate the pattern similarity of these commercial meat products with respect to the different types of meat analyzed in the calibration set (NO-MSM, HP-MSM and LP-MSM). In Fig. 3, it is possible to notice that the validation samples (BT1-10), even if they were analyzed in a later session, are distributed along the cloud of calibration samples analyzed earlier and they are uniformly dispersed among these ones especially along PC1. Thus, the analytical method does not appear to be affected by any systematic error related to the different analytical session. Only the BT8 sample is slightly displaced from the scores cloud

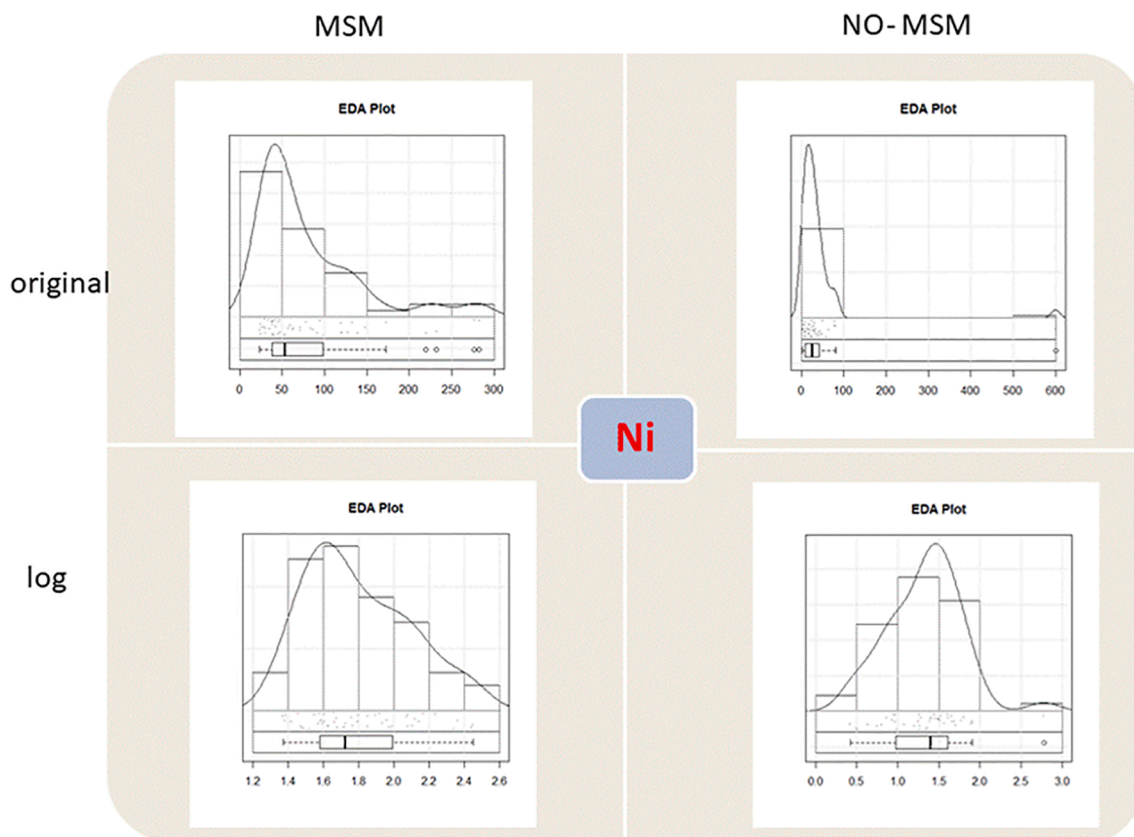


Fig. 1. EDA plots for the variable Ni obtained for the two categories of MSM and NO-MSM samples, before and after logarithmic transform.

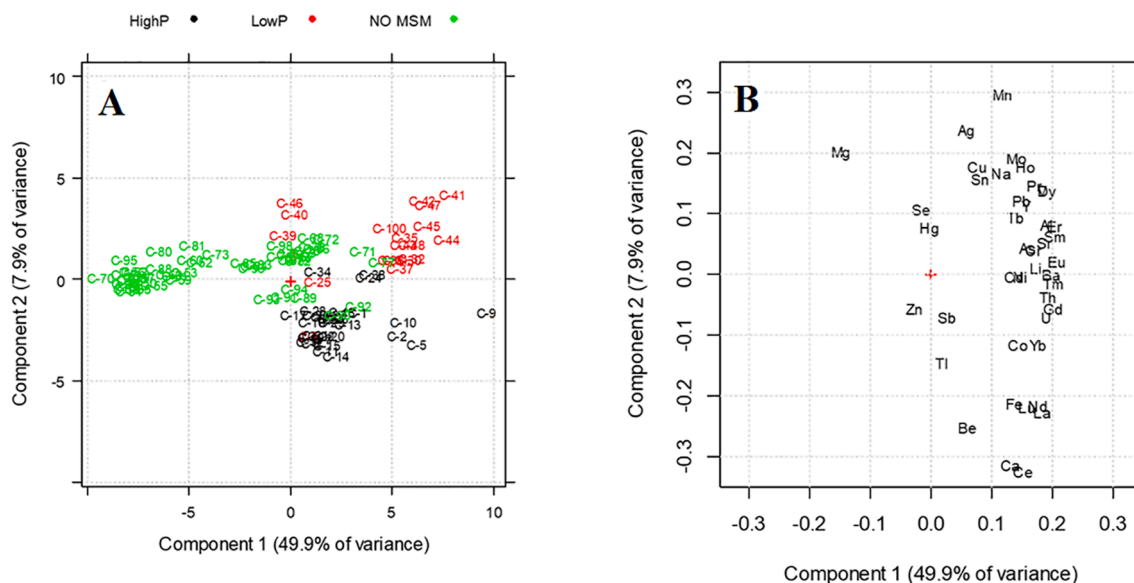


Fig. 2. Score Plot in the space PC1-PC2 (A) and corresponding loading plot (B).

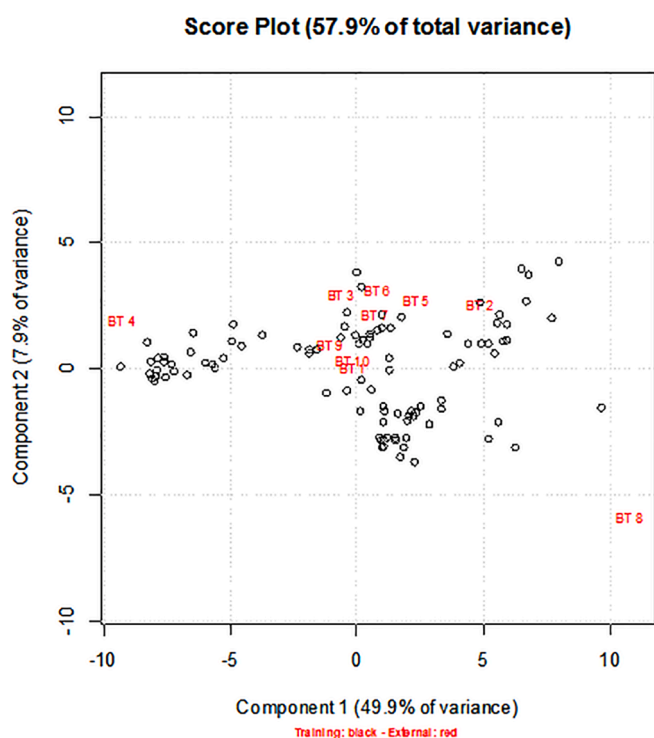


Fig. 3. Projection of the 10 commercial samples into the orthogonal space defined by PC1 and PC2.

along PC2, but being a single sample, systematic errors can be excluded.

3.4 Discriminant analysis

In light of these data processing outcomes, the applicability of a supervised classification technique was demonstrated. Consequently, LDA was performed on the calibration samples and a cross-validation strategy with six cancellation groups was applied for an internal verification of the model reliability. Table 3a shows the results of the discriminant analysis in cross-validation. In particular, the LDA misclassification matrix is reported: the rows are the true classes of the samples and the columns correspond to the class in which the samples

Table 3

Results of LDA classification. Confusion matrix in cross-validation using the 100 meat samples analysed (a); Prediction on 10 unknown commercial meat samples (b).

a	CV results	HP-MSM	LP-MSM	NO-MSM
	HP-MSM	29	1	0
	LP-MSM	1	19	0
	NO-MSM	0	0	50
	Correct Predictions	96.7 %	95.0 %	100.0 %

b	Sample	Predicted category	Real category
	BT 1	HP-MSM	HP-MSM
	BT 2	LP-MSM	LP-MSM
	BT 3	HP-MSM	HP-MSM
	BT 4	NO-MSM	NO-MSM
	BT 5	LP-MSM	LP-MSM
	BT 6	NO-MSM	NO-MSM
	BT 7	NO-MSM	NO-MSM
	BT 9	HP-MSM	HP-MSM
	BT 10	HP-MSM	HP-MSM
	BT 8	NO-MSM	LP-MSM

are assigned by the model. Of the 30 samples in the HP-MSM class, only one was assigned to the LP-MSM class; and similarly, of the 20 LP-MSM samples, only one was assigned to the HP-MSM class. The 50 NO-MSM samples were all classified correctly and, thus, the classification ability in cross-validation for this class was 100 %.

Considering that the null model rate, which is the probability of correctly assigning a sample to the exact class only 'by chance', is 33.3 %, the LDA results can be considered absolutely satisfactory with a total prediction rate in cross validation of 97.2 %.

The last step performed for rigorously validating the model performance was the prediction of the 10 commercial samples. Comparing model outcomes with the analytical determination (performed later), the model was confirmed to be robust and accurate with 9 samples out of 10, which were correctly classified, as reported in Table 3b. Notice that the sample wrongly assigned to the class of NO-MSM (named BT 8), was actually a sample with only 20 % of LP-MSM added to NO-MSM. It is very important to underline that 20 % of MSM is the lowest percentage found on the market during this survey, and that these percentages are often well higher. A thorough diagnostics of the behavior of this sample was performed calculating the Mahalanobis distance between the sample and the centroid of the three different categories, as a measure of

sample compliance with the three classes. As reported in [Suppl. Fig. 4](#), the difference between the three Mahalanobis distances is minimal and this explains the error in classification. This error in classification can be justified by the fact that the sample contained only 20 % of MSM meat, which corresponds to the lowest limit of the calibration range (from 20 to 47 % of LP-MSM in the commercial products analyzed). Taking into account that the lowest percentage of MSM meat in correctly identified LP-MSM samples analyzed during validation was 27 %, it is reasonable to affirm that this is the “limit of detection” which characterizes this analytical approach. Lastly, STEPLDA (with F to enter = 1, F-to-remove 0.5) was applied in order to retain the 15 variables with the highest discriminant power between classes: Ca, Mo, Co, Ba, Sr, Ni, Se, Sn, Ho, Lu, As, Li, Pb, Tl, Fe. LDA was performed on this subset, and a comparison of these results with those obtained with all the 43 variables showed that the discriminant model obtained with 15 variables was equivalent to, or even better than that obtained using all the 43 trace elements. In more detail, a total prediction rate in cross validation of 99.0 %, instead of 97.2 %, was reached.

Finally, the potentialities of this approach are worthy of mentioning. Indeed, this approach was not only conceived for MSM identification in meat products, but also for several other types of determinations in food analysis. For instance, this approach could be developed in food science and technology within research programs aimed at improving the nutritional/organoleptic/sensorial quality of food after modification of product formulation, food processing, packaging, etc. It could be also used for traceability purpose, since the recent advances in this sector of food analysis are based on the simultaneous determination of many analytical parameters and consequent chemometric evaluation.

3.5 Improvement in the field

Most available approaches for the identification of MSM in meat products, as those listed above, allow the clear distinction between HP-MSM and NO-MSM. The most significant drawback of these approaches is the limitation when LP-MSM has to be detected. This limitation is due to the similarity between NO-MSM and LP-MSM under chemical-physical point of view, since the mechanical deboning at low pressure does not lead to significant release of bone fragments, bone marrow, skin, nerves, blood vessels, etc., in meat.

The analytical approach described in this study, on the contrary, can be applied for identifying meat products containing LP-MSM, since it exploits the potential of multi-elemental analysis (43 parameters) coupled to multivariate analysis. This approach allows identifying and highlighting also the smallest differences among different samples, up to discriminate between NO-MSM and LP-MSM. Thus, the analytical method presented, if compared to other available procedures, can be considered as a significant improvement in the field, since it is applicable for official control of meat products when the identification of added MSM is needed.

3.6 Consideration of the green properties

The importance of the eco-friendly aspects in analytical chemistry is well-known. The analytical method reported in this article was evaluated using Analytical GREENness metric approach (AGREE) and Green Assessment Profile Index (GAPI) ([Pena-Pereira et al., 2020](#); [Plotka-Wasyłka, 2018](#)). The AGREE tool is very useful for assessing the environmental and occupational hazards which characterize the analytical procedure. It investigates the 12 principles of green analytical chemistry ([Gałuszka et al., 2013](#)) and gives a final numerical value that represents the level of “greenness” of the method. The final value obtained for the presented method was 0.6 ([Suppl. Fig. 5](#)). Being higher than 0.5, the method can be considered as “green”. This is another important strength of this approach. Furthermore, regarding costs, the present method can be carried out by using a basic single-quadrupole ICP-MS (equipped with a system for reducing interference) whose cost is much lower than the

triple-quadrupole ICP-MS. Regarding mineralization step, it lasts about 90 min performing 15 replicates at the same time (~6 min per sample).

4. Conclusion

In this study, a novel analytical approach for the identification of MSM in different types of meat products was developed and described. Overall, 27 trace elements and 16 rare earth elements were determined in 100 meat samples containing and not containing MSM obtained at low and high pressure, by using ICP-MS. The obtained results were elaborated by means of multivariate analysis and the classification was carried out. The chemometric classification models were rigorously validated, both internally and externally. In details, a cross-validation strategy with six cancellation groups was applied for an internal validation, achieving a total prediction rate in cross validation of 97.2 %. Then, the final validation of this approach was completed by analyzing and classifying 10 ‘blind’ meat samples, obtaining a method accuracy equal to 90 %, representing a significant improvement in field. Thus, the described method is applicable for the discrimination of meat products containing mechanically separated meat, even if the processing was carried out at low and high pressure.

CRedit authorship contribution statement

Oto Miedico: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft. **Valeria Nardelli:** Funding acquisition, Resources. **Teresa D’Amore:** Data curation, Formal analysis, Software, Writing – original draft. **Monica Casale:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Paolo Oliveri:** Data curation, Formal analysis, Methodology, Supervision, Visualization, Writing – review & editing. **Cristina Malegori:** Data curation, Formal analysis, Software. **Giuseppe Paglia:** Data curation, Formal analysis, Data curation, Formal analysis. **Marco Iammarino:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.133842>.

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