



ISTITUTO NAZIONALE DI RICERCA METROLOGICA
Repository Istituzionale

Species-specific detection of processed animal proteins in feed by Raman spectroscopy

This is the author's accepted version of the contribution published as:

Original

Species-specific detection of processed animal proteins in feed by Raman spectroscopy / Mandrile, Luisa; Amato, Giuseppina; Marchis, Daniela; Martra, Gianmario; Rossi, Andrea Mario. - In: FOOD CHEMISTRY. - ISSN 0308-8146. - 229:(2017), pp. 268-275-275. [10.1016/j.foodchem.2017.02.089]

Availability:

This version is available at: 11696/57314 since: 2018-02-19T17:06:56Z

Publisher:

Elsevier

Published

DOI:10.1016/j.foodchem.2017.02.089

Terms of use:

Visibile a tutti

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright

(Article begins on next page)

1 Species-specific detection of Processed Animal Proteins in 2 feed by Raman Spectroscopy

3 Luisa Mandrile^{a,*}, Giuseppina Amato^b, Daniela Marchis^b, Gianmario Martra^a

4 and Andrea Mario Rossi^c

5 ^a *Department of Drug Science and Technology, Università degli Studi di Torino, Via Giuria 9, 10125, Torino, Italy*

6 l.mandrile@inrim.it; gianmario.martra@unito.it

7 ^b *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154, Torino, Italy*

8 Giuseppina.Amato@izsto.it, Daniela.Marchis@izsto.it

9 ^c *Thermodynamic Division, Istituto Nazionale di Ricerca Metrologica, Strada delle Cacce, 91 10135, Torino, Italy*

10 a.rossi@inrim.it

11 ^{*} *Corresponding author: Andrea Mario Rossi, tel +39 011 3919342; fax +39 011 346384; e-mail a.rossi@inrim.it*

12 Abstract

13 The existing European Regulation (EC n° 51/2013) prohibits the use of animals meals in feedstuffs in order to
14 prevent Bovine Spongiform Encephalopathy infection and diffusion, however the legislation is rapidly moving
15 towards a partial lifting of the “feed ban” and the competent control organisms are urged to develop suitable
16 analytical methods able to avoid food safety incidents related to animal origin products. The limitations of the
17 official methods (i.e. light microscopy and Polymerase Chain Reaction) suggest exploring new analytic ways to
18 get reliable results in a short time. The combination of spectroscopic techniques with optical microscopy allows
19 the development of an individual particle method able to meet both selectivity and sensitivity requirements (0.1
20 %w/w). A spectroscopic method based on Fourier Transform micro-Raman spectroscopy coupled with
21 Discriminant Analysis is here presented. This approach could be very useful for in-situ applications, such as
22 customs inspections, since it drastically reduces time and costs of analysis.

23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

keywords: PAPs, Raman spectroscopy, species identification, chemometrics

1. Introduction

The outbreak of bovine spongiform encephalopathy (BSE) urged the European Union to take several decisions in order to avoid the transmission of its most probable causal agent through the food chain, which is a protein residue called prion. It is generally accepted that the most likely route of infection of cattle with BSE is by feeding cattle with infected processed animal proteins (PAPs). The commercially available PAPs appear as grinded dry meals and consist into two main fractions: i) the lightweight fraction, including muscle fibers, hairs and grease and ii) the heavy residual, containing the mineral fraction, bone fragments, tooth fragments, cartilage etc. The two fractions can be easily separated through a sedimentation procedure using high density solvent such as tetrachloroethylene. PAPs from different animal origin have peculiar features in terms of color and smell. mammalian PAPs are characterized by a high fraction of hard bones fragments, typical for high-size animals' skeleton structure. PAPs derived from swine slaughter processes has light ivory color, is very finely grinded and the unsaturated fat content is higher with respect to ruminant PAPs. Poultry and fish meal are characterized by darker color, pungent smell, rough grinding and the total fat content is mostly represented by saturated oils; the bones fragments in this case are mainly soft bones tissues with high cartilaginous content.

The legislation about the prevention, control and eradication of BSEs evolved a lot during the past 15 years through several EC regulations (Van Raamsdonk 2007). Particularly relevant are Regulation (EC) n° 999/2001 and Regulation (EC) n° 1069/2009 (Animal by-products Regulation) which prohibits the feeding of terrestrial animals of a given species other than fur animals with PAPs derived from the bodies or parts of bodies of animals of the same species (ban of intra-species recycling). After the publication of the TSE Road Map II (rev. 10) in 2010, a lifting of the ban about the use of PAPs from non-ruminants in non-ruminant feed could be considered, of course without lifting the existing prohibition on intra-species recycling. The most recent European Regulation (UE) n°56/2013 highlights that the availability of effective and validated tests to distinguish between PAP of different animal species should be a prerequisite of any possible reintroduction of non-ruminant PAP to feed other non-ruminant cattle, as well as a careful analysis of the risks of relaxation, regarding animal and public health. In other words the feed-ban lifting would turn into a revision of the present legislation, only if validated and accredited analytical techniques to determine the species origin of PAP will be available and if an efficient channeling of PAPs from different species will be in place.

51 In this concern, in 2012, the European Union Reference Laboratory for Animal Proteins in feedstuffs (EURLAP)
52 validated a new Polymerase Chain Reaction (PCR) method which is able to detect very low level of ruminant
53 material that may be present in feed and a validated method to detect porcine DNA is on the way. Official
54 methods for the detection of ruminant DNA and optical microscopy lay down in Regulation (EU) 51/2013.
55 Thanks to these official methods the feed-ban was partially lifted, and non-ruminant PAP are now allowed in
56 fish feed. The microscopy method is based on the recognition of morphological characteristics of bone
57 fragments, or other structures, e.g. muscle fibers, animal fur, teeth fragments, feathers etc. The fine image
58 analysis using 10X, 20X and 40X objective magnifications allows PAP from terrestrial to aquatic animals to be
59 distinguished in accordance with the osteocytes' *lacunae* morphology and bones peculiarities. Nevertheless, the
60 confusing features revealed between mammalian and poultry PAPs do not allow a correct discrimination of the
61 two classes using optical microscopy. On the other hand, PCR provides high sensitivity and specificity but it
62 does not reveal the nature of the DNA trace sources. This represents a problem since ruminant DNA is present
63 both in authorized (i.e. milk) and in not authorized (i.e. ruminant PAP) ingredients in feed. Another lack of the
64 official methodologies is that they do not allow the quantification of animal origin constituents in feed. Both
65 methods have a declared cut-off concentration around 0.1 % (w/w).

66 Other non-official methods, such as near-infrared spectroscopy (NIRS) (Murray, Aucott and Pike 2001;), near-
67 infrared microscopy (NIRM) (Baeten et al 2005, Pavino et al 2009), liquid chromatography (LC) and olfactometry
68 techniques were tested for the detection of PAPs in literature. LC methods are based on the detection of specific
69 polypeptides. For example, carnosine is mainly present in mammals and anserine is mainly found in birds
70 (Schönherr 2002). On the other hand, LC methods are difficult to standardize, because polypeptides are usually
71 strongly denatured because of the high temperature treatments required by Annex IV of Regulation (EC)
72 142/2011. Olfactometry is based on the detection of volatile non-specific agents (Campagnoli et al 2004) and it
73 represents an interesting non-destructive and fast technique. Unfortunately, it turned out that the presence of fish
74 material could mask the detection of proteins of land animals, even at a contamination level of 5 g/kg.
75 Spectroscopic techniques are non-destructive methods that can be applied for *in situ* analysis in feed production
76 plants or in farms, but the too high detection limit did not allow their diffuse application in official control
77 laboratories yet. Anyway, the combination of spectroscopic techniques with optical microscopy could lead to an
78 individual particle method, and reach the sensitivity required. Near and mid infrared spectroscopy, as well as
79 Raman spectroscopy, are promising techniques because of their molecular specificity. The complex pattern of

80 vibrational peaks contains a lot of chemical information and it represents the sample's molecular fingerprint. A
81 spectroscopic approach based on Fourier Transform Raman spectroscopy (FT Raman) for detection of PAPs in
82 feeds is here presented. Moreover, a specific method based on the multivariate treatment of spectra for species
83 recognition is shown and the principal advantages and disadvantages of this method are addressed in this work.
84 In the big-data era modeling techniques are widely exploited to describe complex systems in many fields of
85 research and for different applications (Valipour, Singh 2016). The aim of this study is to demonstrate that a
86 multivariate classification models can provide a consistent tool for sample recognition, even if matrices are
87 complex as feedstuffs and PAPs are. The major advantages of the proposed method, with respect to the
88 established optical microscopy and PCR methods, are the reduction of time and costs of analysis, the automation
89 and the objectivity of results, because of the low operator-dependence of Raman spectroscopy. After setting the
90 classification model, a minimal time is required to operators to launch the Raman mapping analysis and no
91 highly qualified operators are needed. The combination of spectroscopic techniques with optical microscopy and
92 chemometrics permits to develop a highly sensitive and specific method. The molecular fingerprint collected
93 using vibrational spectroscopy represents one interesting way towards the species specific-method, strongly
94 required for the further European Regulations about intra-species recycling controls.

95 **2. Material and Methods**

96 *2.1 Reagents and materials*

97 Pure PAPs sample of category 3 (PAPs derived from animals suitable for human consumption) from four
98 different species (bovine, swine, poultry and fish) and a generic feed were provided by the Veterinary Medical
99 Research Institute for Piemonte, Liguria and the Valle D'Aosta (IZSTO). Tetrachloroethylene (specific gravity
100 $1,62 \text{ g/cm}^3$) was purchased from Nova Chimica (Milan, Italy). Conical glass separation funnels with a content of
101 250 ml with Teflon or ground glass stopcock at the cone base were used for sedimentation procedure. Stopcock
102 opening diameter $\geq 2 \text{ mm}$ was used for sedimentation procedure. Laboratory press for tablets preparation with
103 dial indicator to enable reproduction of force settings (E-Z™ Quick Table Top Press) was provided by
104 International Crystal Laboratories, and it was used to prepare $\approx 0.3 \text{ mm}$ thick tablets.

105 *2.2 Sample preparation*

106 The material was carefully mixed to obtain a homogenized sample. Pure PAPs samples were already milled,
107 whereas vegetal feed in pellets was grinded in a laboratory blender up to granulometry <0.5 mm. All reusable
108 equipment was carefully cleaned before use to avoid laboratory cross-contamination. Separation funnel pieces
109 and glassware were pre-washed manually and cleaned by using a brush with stiff synthetic hairs. A final
110 cleaning of sieves with acetone and nitrogen flux was recommended.

111 For the calibration set construction pure PAPs were used. The method sensitivity was evaluated through
112 standards meal samples with a known contaminant concentration. A commercial feed, previously tested with
113 classical microscopy method to exclude PAP contamination, was fortified with known amount of PAPs, ranging
114 from 0.1 to 10 % w/w. A reference sample spiked with PAP at 0.1 %w/w was prepared by the EURL-AP for
115 inter laboratory study and provided by the Italian Reference Laboratory (IZSTo).

116 In accordance with Regulation (EU) 51/2013 extraction and preparation of the sediment, a portion of 3 g of pure
117 PAP (accuracy of 0.01 g) of the ground sub-sample was transferred into the separation funnel or conical
118 bottomed settling beaker and 50 ml of tetrachloroethylene were added. The mixture was vigorously shaken for at
119 least 30 s and at least 50 ml more of tetrachloroethylene were added cautiously while washing down the inside
120 surface of the funnel to remove any adhering particles. For the contaminated feed analysis 10 g of feed was used
121 and two aliquots of 50 ml of tetrachloroethylene for separation. Both resulting mixtures were left to stand for at
122 least 5 minutes before the sediment were separated off by opening the stopcock and dried on a paper filter.
123 Sediment fraction was about 10 % of the whole sample of pure PAP.

124 *2.3 Scanning electron microscopy and EDX*

125 Scanning Electron Microscopy images of bone fragments were collected using Inspect F (FEI, Hillsboro,
126 Oregon), acceleration potential used was 10 kV. Samples were covered with 10 nm of Au/Pd alloy using
127 sputtering technology. Energy-dispersive X-ray spectroscopy was performed using Inspect F (FEI, Hillsboro,
128 Oregon), as well.

129 *2.4 Raman measurement*

130 Thin self-consistent tablets for Raman analysis were prepared using a laboratory press. This method is very rapid
131 and safe in comparison with microscope glasses preparation since solvents and drying time are avoided. This is a
132 very useful condition for Raman imaging, as in flat tablets all fragments lied exactly at the same distance from

133 the laser source, and each spectrum of a Raman map can be collected in suitable focus conditions. In this way
134 focus was set only once before the Raman mapping analysis. Raman spectroscopy analysis was performed with a
135 NXR FT-Raman Module Nicolet Series™ (Thermo Fischer Scientific, Waltham, USA) equipped with an
136 InGaAs detector. The laser power of 0.8 W was used for the calibration set collection, whereas 0.3 W was used
137 for the Raman mapping of contaminated feed samples. Raman spectra collection was optimized using 1064 nm
138 excitation laser mounted on a Fourier Transform Raman instrument to minimize the fluorescence phenomena in
139 competition with Raman signal. The wavenumbers range considered was $200\text{ cm}^{-1} - 4000\text{ cm}^{-1}$. A resolution of 8
140 cm^{-1} was applied, and 256 scans were collected for each map point to obtain a S/N ratio higher than 10. For rapid
141 screening tests fast Raman mapping was performed with 1 mm diameter laser spot (instead of $50\text{ }\mu\text{m}$) and 64
142 scans for each map point, instead of 256.

143 *2.5 Calibration procedures*

144 The raw Raman spectra were treated for chemometric analysis by TQ Analyst™ 8.0 software. A preliminary
145 correlation test was performed to attest a correlation between the spectroscopic features (variables) and the
146 belonging class (responses). Then a consistent data set was collected so that the total variability of the system is
147 described; then the discriminant analysis (DA) method was applied, to obtain a robust classification model. All
148 calibrated models were validated through an external validation procedure by new test set.

149 **3. Results and Discussion**

150 *3.1 Optical and electronic microscopy*

151 The limits of the official optical method for identifying PAPs in feedstuffs suggest exploring new analytic ways
152 to get more information from samples with simple and quick techniques. The heterogeneity of the matrices and
153 the low limit of detection required by the legislation into force (0.1 %), represent the most challenge in setting an
154 easy and fast method of analysis. In this study a “bottom up” approach was carried out in order to hit the target.
155 First, pure analytes (i.e. the PAPs from different animal species) were analyzed, to prove that Raman technique
156 was able to characterize the PAPs with high specificity. Interferences of feed materials of vegetal origin were
157 ignored at first.

158 The fraction of sedimented PAPs in TCE can represent variable percentage of the whole sample weight
159 depending on the species (for instance, processed bovine proteins have a higher sedimentable fraction compared
160 to processed swine proteins). The most part of sediment contains bone fragments as demonstrated by and EDX

161 analysis (Energy-dispersive X-ray spectroscopy), which revealed the typical composition of mineral biological
162 tissue containing Ca, P, Na, O, H.

163 The optical identification of animal fragments in the sediment fraction is based on the systematic observation of
164 fragments lied on a microscope glass using 10X, 20X and 40X objectives. Optical images collected in
165 transmission mode of fish and terrestrial animal bone's fragments are shown in Fig. 1. In the optical images
166 osteocytes *lacunae* can be easily identified as well as the *lacunae* ramification. The shape and size of the
167 osteocytes' *lacunae* and their ramifications are the most specific features for the discrimination of PAPs of
168 aquatic and terrestrial animals. Bone fragments are stained with red alizarin dye to facilitate the identification, as
169 suggested in the official procedure. However, this recognition can be lead only by well-trained operators and, in
170 any case with some level of uncertainty.

171 Fig.1

172 More accurate information about bone fragments morphology is collected by Scanning Electron Microscopy to
173 investigate fine structural differences among different animal species. Unfortunately, after the grinding process
174 bone fragments show a damaged structure; SEM images of bone fragments permit to identify the structural
175 components of bones described in literature. In particular the most common building blocks of bones such as
176 collagen fibrils, mineral plates, un-mineralized matrix, non-fibrillar organic material (mostly made of
177 proteoglycans and glycoproteins) can be identified using SEM. (Turner et all 2006 a; Turner et all 2006 b). For
178 example, SEM images of bone fragments from different species shown in Fig. 2a represent fibrils coated with a
179 large amount of non-fibrillar organic material. In Figure 2b osteocytes *lacunae* can be identified with a good
180 perception of their 3D structure.

181 Fig.2

182 Unfortunately SEM images do not reveal to be more informative than optical microscopy in this domain. It is not
183 possible to identify very characteristic structures that could be univocally associated to one animal species. All
184 PAPs samples analyzed with SEM presented fragments with similar morphologies and it was not possible to
185 identify some very specific indicator that allows a certain classification of bones from different animal species.
186 Even if it is not excluded that an accurate study using powerful images analysis techniques could provide some
187 interesting results in the future. However, neither recognition software, nor complete libraries are now available
188 about bones fragments using SEM. What is more, it seems to be worthless to use such an expensive technique
189 for morphological analysis, the procedure is time consuming and unsuitable for automation. Therefore, even if

190 well trained operators could perform the microscopic official methods rather easily, it is not possible to indicate
191 SEM as a robust method for animal species recognition in feed.

192 *3.2 Raman characterization of meat and bone meals*

193 Automatic spectrometer coupled with microscopy technology could represent a valuable tool for implementing
194 the existing methodology. In this way morphological information provided by the microscope and chemical
195 information provided by Raman spectroscopy could be merged in order to obtain the best recognition of animal
196 fragments and their species of origin. Raman characterization of the two separated fractions (i.e. sediment and
197 flotata) revealed that the chemical nature and optical and physical features of the flotata fraction make it difficult
198 to collect Raman spectra. Fluorescence phenomena and the huge heterogeneity of samples would represent a
199 great limitation for a possible routine Raman analysis for PAPs. Otherwise, the sediment resulted to be more
200 suitable for Raman characterization. Sediment fraction of PAPs was particularly resistant to laser power,
201 therefore 0.8 W laser power was set for pure PAPs analysis; this permits to reach S/N ratio out of 15 with 256
202 scansions. Random variability of spectra of different fragments of the same PAP sample involved only the
203 background intensity of the entire spectra; no differences, neither in mutual intensity of peaks nor in peaks'
204 shape were recorded. A normalization procedure of spectra revealed that spectra of the same species perfectly
205 overlapped. Signals can be assigned to bone and meat chemical components. In fig. 3 a model spectrum of PAP
206 is shown with the attribution of the Raman bands to the corresponding chemical functional groups (Socrates
207 2001). The attention was focused on the band at 960 cm^{-1} due to hydroxyapatite compound (Figueiredo, Gamelas
208 and. Martins 2014) which is characteristic for bone's fragments. This signal is suitable as reference signal and it
209 could be successfully used as an "alert signal" during screening analyses. The presence of such peak could
210 therefore represent the very first warning about a PAP contamination in the sample.

211 Fig 3.

212 The spectra profile was very similar for all tested species. Differences among species are basically in shape,
213 mutual intensity and baseline trend of spectra as it can be noticed in the Fig 3b.

214 *3.3 Multivariate data treatment and classification*

215 Since spectra of different PAPs look very similar, a monivariate analysis of a single Raman peak can not lead to
216 any significant result. Contributions of both the organic and the inorganic components result in a characteristic

217 pattern of spectroscopic signals, which can be interpreted only through a multivariate approach. Calibration
218 procedure requested the preliminary collection of a training set made of about 80 samples spectra; than
219 discriminant analysis method was applied to obtain a classification model.

220 No consistent separation was achieved when all species (bovine, swine, fish, and poultry) were considered
221 contemporarily. Unfortunately, any optimization such as mathematical pretreatment and variable selection was
222 straightforward for a classification model composed of four classes. The test set validation failed for 25% of
223 validation spectra in the best case, therefore the simultaneous classification of all species was excluded and a two
224 steps procedure was developed. Taking into account preliminary observation made on the data set, a two class
225 model was developed: initial separation of mammals (i.e. bovine and swine) from non-mammals (i.e. fish and
226 poultry) animals was set, in accordance with visible spectra similarity. Appreciable improvements in
227 classification model were obtained by setting a model composed of two classes: i) mammals (bovine and swine)
228 and ii) non mammals (fish and poultry) (Fig. 4a). The obtained DA model was calculated considering 10
229 principal components, which correspond to a cumulative explained variance of 95 %. All spectra were treated by
230 standard normal variate (SNV) for minimizing pathlength differences. The method underwent two different
231 validation tests: leave-one-out cross validation as an internal validation procedure provided fully correct
232 answers. Then external validation test was performed using an external test set. Also in this case no incorrect
233 classification was obtained.

234 A second classification step was then added to recognize the single species. Other two dual class models were
235 optimized to discriminate bovine from swine meal (Fig 4b) and fish from poultry meal (Fig 4c) using a similar
236 strategy. Fish/poultry separation model gave satisfying results; 8 PCs were used, corresponding to a cumulative
237 explained variance of 97 % and 0% of misclassified during external validation were obtained. Otherwise,
238 bovine/swine model resulted to be harder to be optimized, as the variability between swine and bovine spectra
239 was slighter. Anyway 0% misclassified of validation samples were obtained using 6 PCs which show a
240 cumulative explained variance of 94%. Thus, a two steps procedure for an exhaustive specific recognition of
241 animal origin of PAPs was implemented and validated for pure PAPs samples.

242 Fig. 4

243 *3.4 Set up of the innovative analytical methodology*

244 In order to test the applicability of the proposed analytical method for the identification of PAPs in feeding stuff
245 an analytical procedure was developed and assessed. The micro Raman method proposed was built on the basis
246 of official optical microscopy method, and paves the way to a possible automation of the procedure,
247 implementing an automatic preliminary scan using Raman imaging. A procedure scheme of the whole innovative
248 analytical method is shown in Figure 5. The automatic Raman imaging enables the operator to manage his
249 working time in a more efficient way, leaving to the instrument the commitment of scanning the samples looking
250 for the animal fragments. Moreover, a species- specific analysis could be carried out.

251 Figure 5.

252 Since high quality spectra collection would require a few minutes and the area to be analyzed is about 0.5 cm^2 ,
253 the time of analysis would be too long for routine applications. Therefore, the analytical method was split into
254 two phases: i) screening step, by fast Raman mapping of the whole tablet's area with $500 \mu\text{m}$ step-size, 1 mm
255 laser spot and 64 scansions for each map point, as mentioned in M&M section, for preliminary suspect
256 fragments identification; ii) a more accurate spectrum collection, suitable for species recognition. After
257 collection, the screening map was profiled according to the Raman intensity at 960 cm^{-1} , which represents a
258 specific peak for bones. This peak is usually intense, sharp and visible even in fast spectra collection. A color
259 scale related to 960 cm^{-1} intensity was associated to the x-y position on the map, to recognize suspect fragments
260 (Fig. 6). After the entire map collection the operator was requested to lead the laser spot on those alert points,
261 using the powered sample stage, and collects new high quality spectra for species identification.

262 Fig. 6

263 The method sensitivity was tested using a reference sample contaminated with PAP at 0.1% w/w which is the
264 currently accepted limit of detection (LOD). The reference sample was prepared by the EURL-AP for an inter
265 laboratory study and provided by IZSTo. The proposed Raman imaging method allowed the identification of
266 PAPs up to 0.1 % the method was set as a reiterative method. The sample can be declared "PAP-free" after six
267 Raman map collections. The low resistance of sediments to high laser power used for species-specific calibration
268 models and the interferences due to feed matrices during the high specific spectra collection may affect the
269 results. Nevertheless, these two issues did not lead to any mislead when the screening map was acquired with 0.3
270 W laser power and for PAP's fragments of dimensions upper then $100 \mu\text{m}$.

271 **4. Conclusions**

272 In this work a method to identify the presence of PAPs in feed was developed and validated, as well as a species-
273 specific analysis for the classification of PAPs found in contaminated feed. The suitability of Raman
274 spectroscopy for the automatic identification of PAPs contamination in feed up to the existing LOD was
275 demonstrated. The most sources of error of this work were related to the meal granulometry and matrix
276 heterogeneity which made it difficult to reach very low sensitivity. These drawbacks could be solved adding at
277 the top of the analytical procedure a grinding, homogenization and sifting step to obtain a more suitable powder
278 for accurate contamination analysis. The validated Raman method is supposed to represent a semi-automatic
279 screening method for intra-species recycling control, to support optical microscopy official method. All the
280 analyses carried out in this study allows us to conclude that the combination of the sensitivity to molecular
281 structure of Raman spectroscopy with optical microscopy can result in an effective method for the detection of
282 PAP fragments in feedstuffs in a green-chemistry view. This method provides not operator dependent and time
283 effective response. Indeed, after setting the classification model, a minimal time is required to operators to
284 launch the sediment Raman mapping analysis and no high-qualified operators are needed. With this respect,
285 future perspectives of this work are the revalidation of the proposed methodology directly in the control
286 organisms' laboratories and inter-laboratory comparison studies to attest the wide scale reproducibility of the
287 Raman methodology for the detection of animal origin contamination in feed. Moreover, in the future a similar
288 analytical approach will be tested to promote a green analytical method also for new feed components such as
289 insect meals. This automatic approach could be very useful for on-site applications, such as product official
290 controls at the EU borders, as well as for custom inspections. In particular, avoiding sample delivery to a trained
291 laboratory, time and costs of analysis would be drastically reduced.

292 **Acknowledgements**

293 The present research was found by "Ricerca Corrente 2013" project; id number IZS PLV 08 /13 RC

294 **References**

295 V. Baeten, C. Von Holst, A. Garrido, J. Vancutsem, A. Renier Michotte, P. Dardenne, "Detection of banned meat and bone
296 meal in feedstuffs by near-infrared microscopic analysis of the dense sediment fraction." *Anal. Bioanal. Chem.* **382**, 149–157
297 (2005)

298 A. Campagnoli, L. Pinotti, G. Tognon, F. Cheli, A. Baldi, V. Dell'Orto "Potential application of electronic nose in processed
299 animal proteins (PAP) detection in feeding stuffs *Biotechnol.*" *Agron. Soc. Environ.*, **8** (4), pp. 253–255 (2004)

300 M.M. Figueiredo, J.A.F. Gamelas and A.G. Martins, Characterization of Bone and Bone-Based Graft Materials Using FTIR
301 Spectroscopy, *Infrared Spectroscopy - Life and Biomedical Sciences*, Prof. Theophanides Theophile (Ed.), ISBN: 978-953-
302 51-0538-1 (2012)

- 303 I. Murray, L.S. Aucott and I.H. Pike, "Use of discriminant analysis on visible and near infrared reflectance spectra to detect
304 adulteration of fish meal with meat-and-bone meal." *J. Near Infrared Spectrosc.*, **9**, 297-311 (2001)
- 305 D. Pavino, S. Squadrone, M. Cocchi, G. Martra, D. Marchis, M.C. Abete, *Food Chemistry* 121 (2010) 826–831
306 doi:10.1016/j.foodchem.2009.12.092
- 307 J. Schönherr, "Analysis of products of animal origin in feeds by determination of carnosine and related dipeptides by high-
308 performance liquid chromatography" *J. Agric. Food Chem.*, **50**, pp. 1945–1950 (2002)
- 309 George Socrates, *Infrared and Raman Characteristic Group Frequencies*, Third edition WILEY ed. 2001
- 310 P.J. Thurner, B. Erickson, Z. Schriock, J. Langan, J.Scott, M. Zhao, J. C. Weaver, G. E. Fantner, P. Turner, J.s H. Kindt, G.
311 Schitter, D. E. Morse, P. K. Hansma, "High-Speed Photography of the Development of Microdamage in Trabecular Bone
312 During Compression" *Journal of Materials Research*, (2006)
- 313 P. Thurner, B. Erickson, Z. Schriock, J.C. Weaver, G.E. Fantner, G. Schitter, J.H. Kindt, P. Turner, D.E. Morse, P.K.
314 Hansma, "High Speed Photography of Compressed Human Trabecular Bone Correlates Whitening to Microscopic Damage",
315 American Control Conference 2006
- 316 L.W.D. van Raamsdonk, C. von Holst, V. Baeten, G. Berben, A. Boix, J. de Jong, New developments in the detection and
317 identification of processed animal proteins in feeds, *Animal Feed Science and Technology*, **133**, Issues 1-2, Pages 63–83
318 (2007)
- 319 M. Valipour, V. P. Singh 2016, B. Maheshwari et al. (eds.), *Balanced Urban Development: Options and Strategies for
320 Liveable Cities*, Water Science and Technology, Library 72, DOI 10.1007/978-3-319-28112-4_18
- 321 COMMISSIO REGULATION (EU) No 56/2013. URL [http://eur-lex.europa.eu/legal-](http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0056&qid=1477574827733&from=EN)
322 [content/EN/TXT/PDF/?uri=CELEX:32013R0056&qid=1477574827733&from=EN](http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0056&qid=1477574827733&from=EN). Accessed 27.10.2016
- 323 Validation study of a real-time PCR method developed by TNO Triskelion bv for the detection of ruminant DNA in
324 feedingstuffs. URL <http://eurl.craw.eu/index.php?page=24&id=10>. Accessed 27.10.2016
- 325 COMMISSION REGULATION (UE) 51/2013. URL [http://eur-lex.europa.eu/legal-](http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0051&rid=2)
326 [content/EN/TXT/PDF/?uri=CELEX:32013R0051&rid=2](http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0051&rid=2). Accessed 27.10.2016
- 327 REGULATION (EC) No 1774/2002 of THE EUROPEAN PARLIAMENT AND OF THE COUNCIL. URL [http://eur-](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2002R1774:20070724:EN:PDF)
328 [lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2002R1774:20070724:EN:PDF](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2002R1774:20070724:EN:PDF). Accessed 27.10.2016
- 329 <http://hansmalab.physics.ucsb.edu/afmbone.html>. Accessed 27.10.2016

330

331 **FIGURE LEGENDS**

332 **Figure 1**– a,b) terrestrial bone; c) fish bone; d) fish cartilage images. All images are purchased by IZSTO.

333 **Figure 2**– a) Bovine (fibrils coated by non-fibrillar organic material); b) fish (osteocyte holes); c) poultry
334 (pressure fracture in bone tissue); d) swine bones fragment.

335 **Figure 3**– a) typical Raman spectrum of PAP sediment with the characteristic signals assignments. The
336 hydroxyapatite peak, relevant for further analysis is highlighted in a rectangular box. b) FT-Raman spectra of
337 PAPs from swine, bovine, fish and poultry compared, the scale is normalized.

338 **Figure 4**– scores plot of optimized separation models for a) bovine-swine vs. fish-poultry; b) swine vs. bovine;
339 c) fish vs. poultry.

340 **Figure 5**– Analytical method scheme of identification and recognition of PAPs in feedstuffs.

341 **Figure 6**– Raman mapping of feed tablet contaminated at 0.1 % with bovine PAP.

342

343

344

345

Figure 1
[Click here to download high resolution image](#)

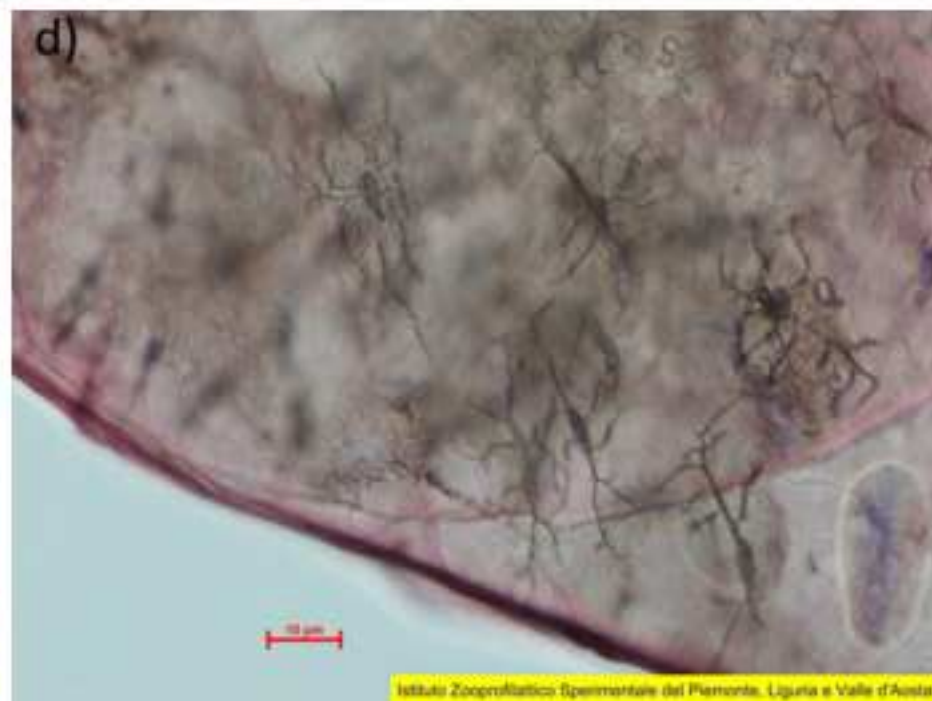
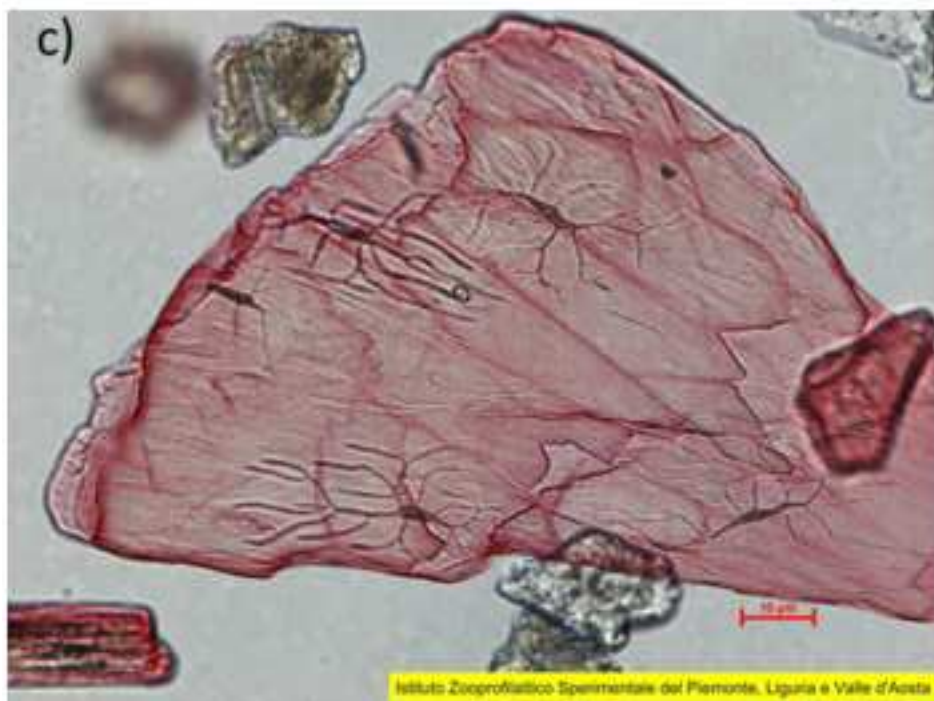
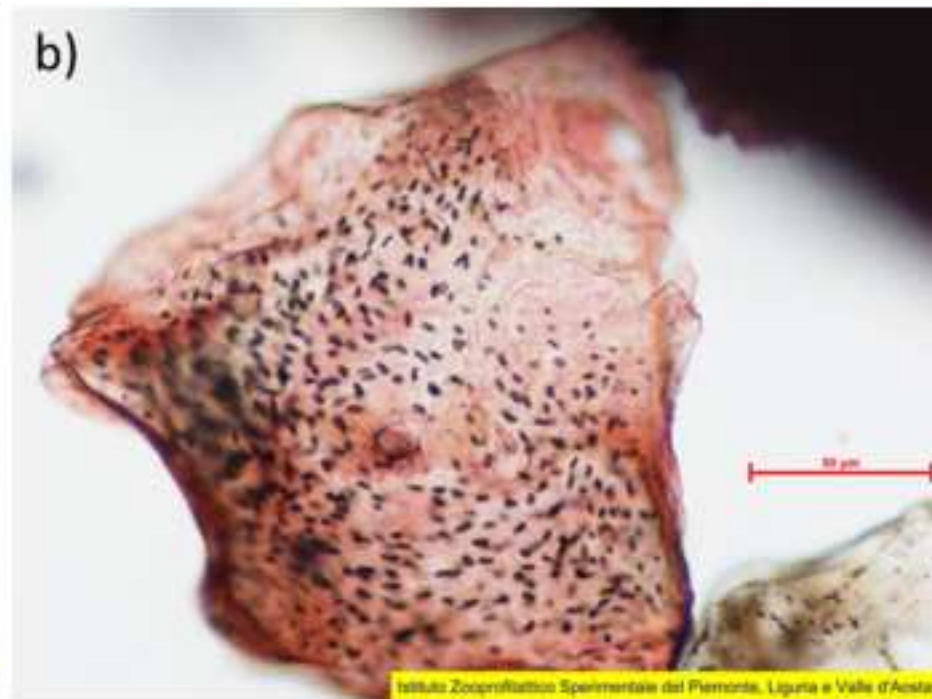


Figure 2
[Click here to download high resolution image](#)

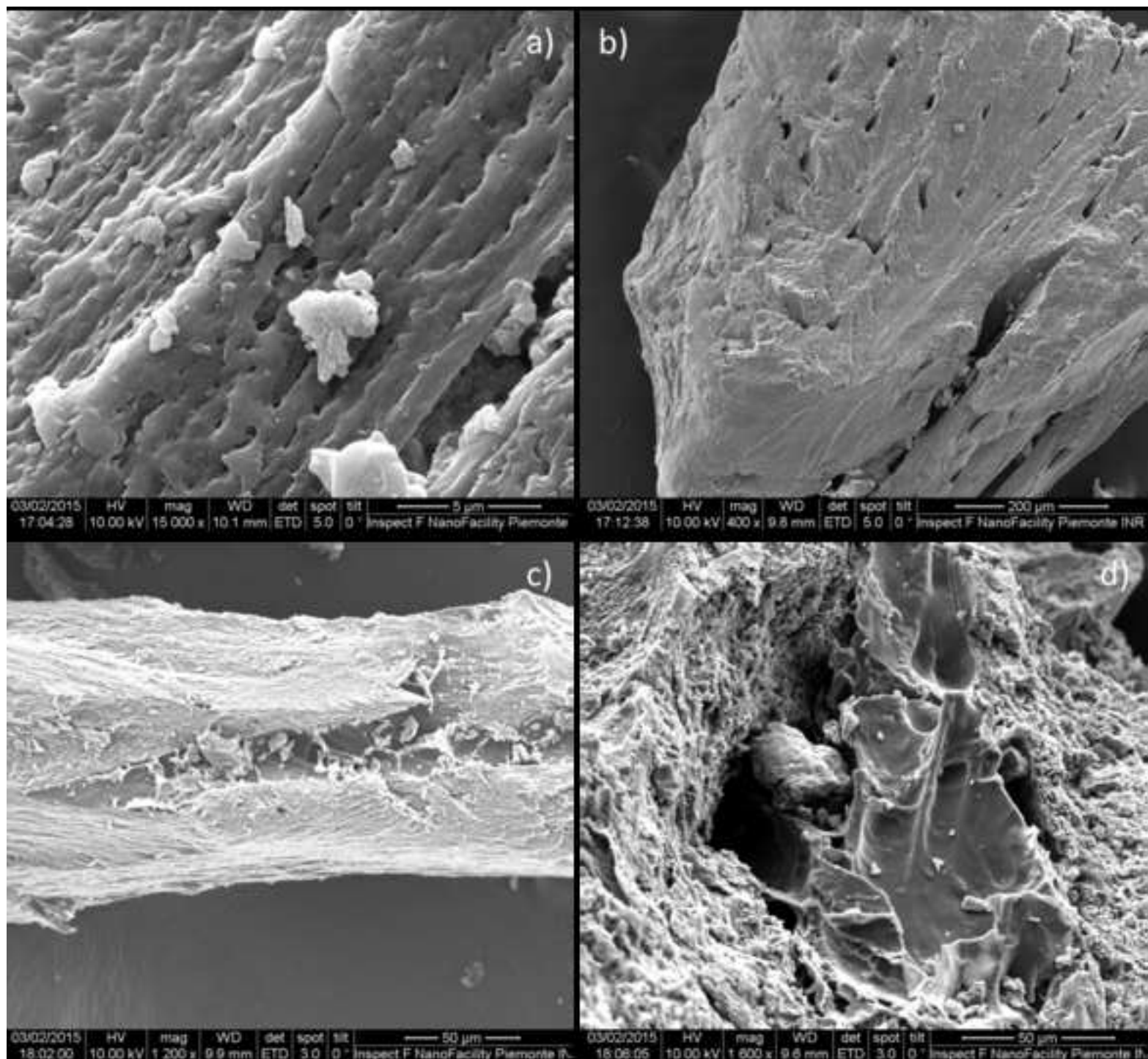


Figure 3
[Click here to download high resolution image](#)

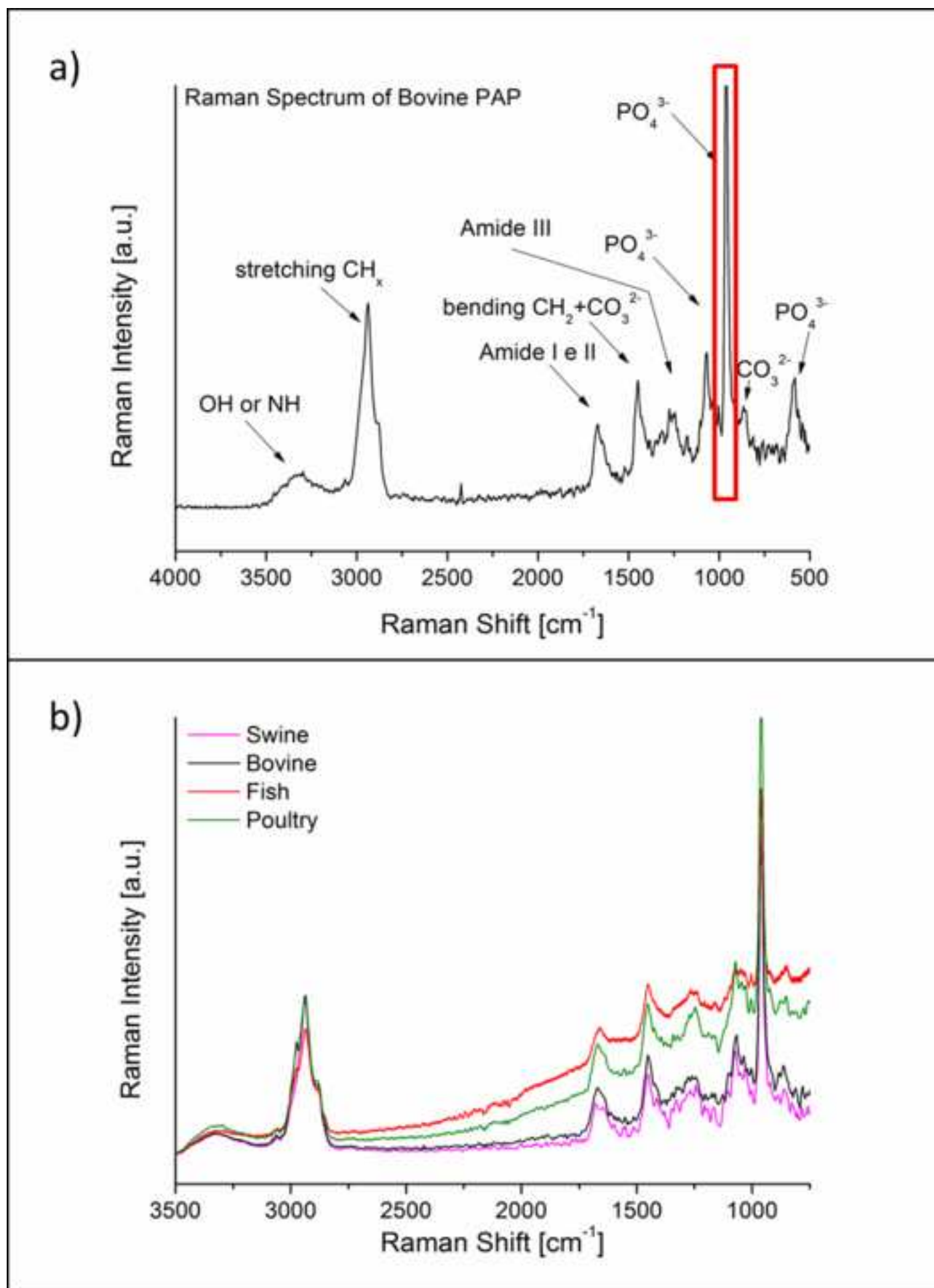


Figure 4
[Click here to download high resolution image](#)

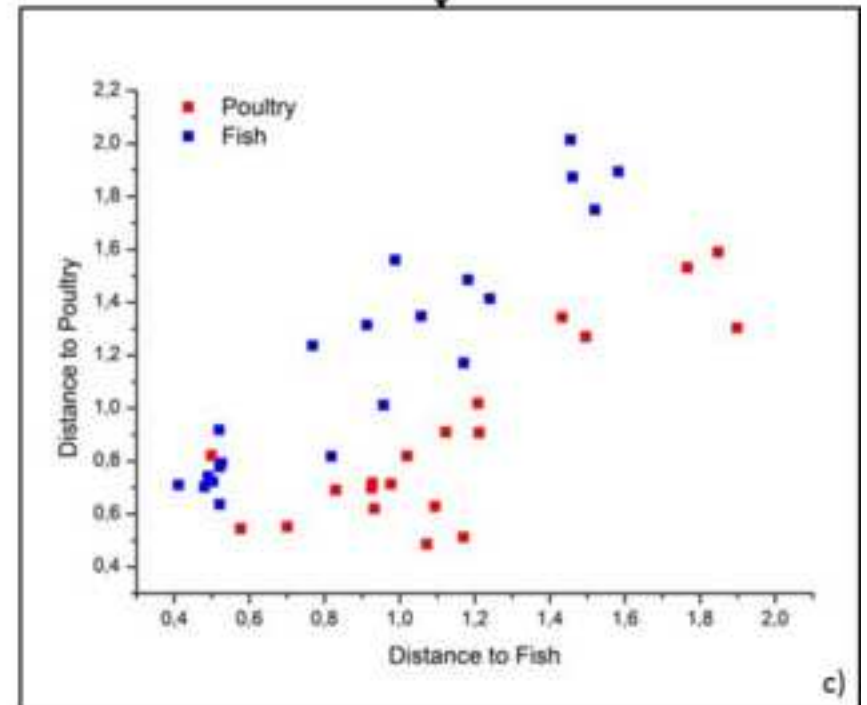
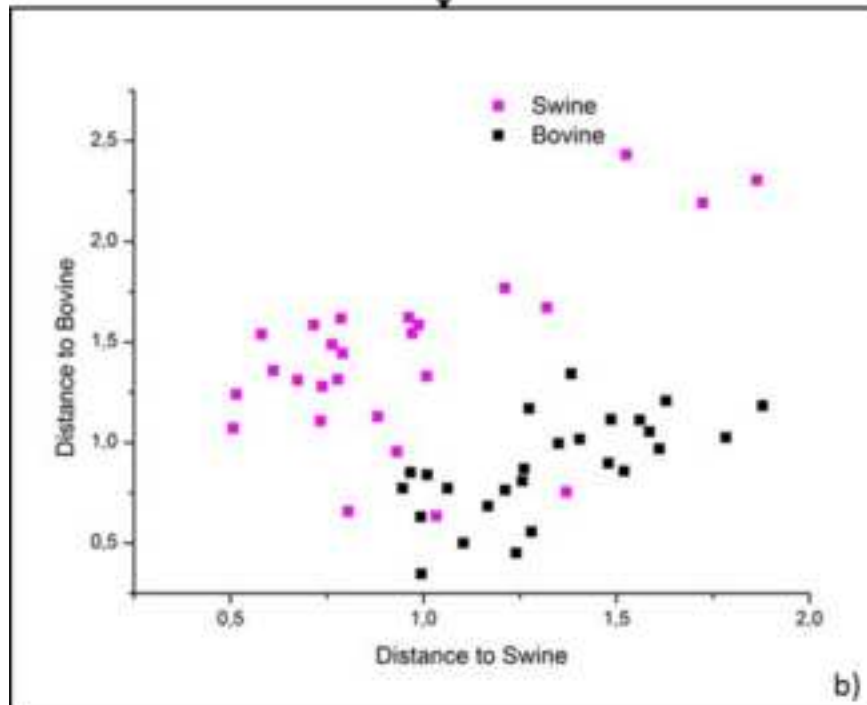
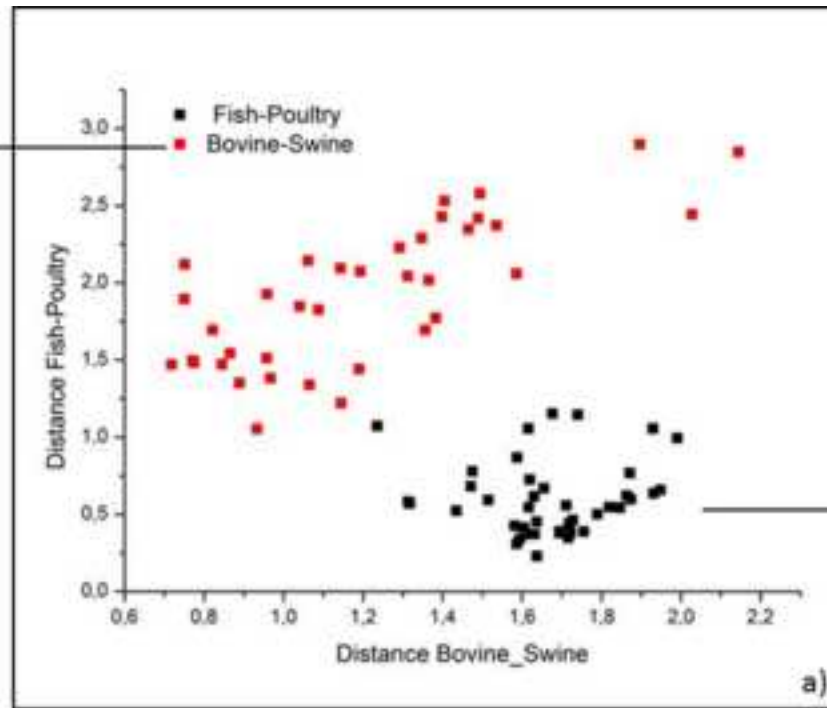


Figure 5
[Click here to download high resolution image](#)

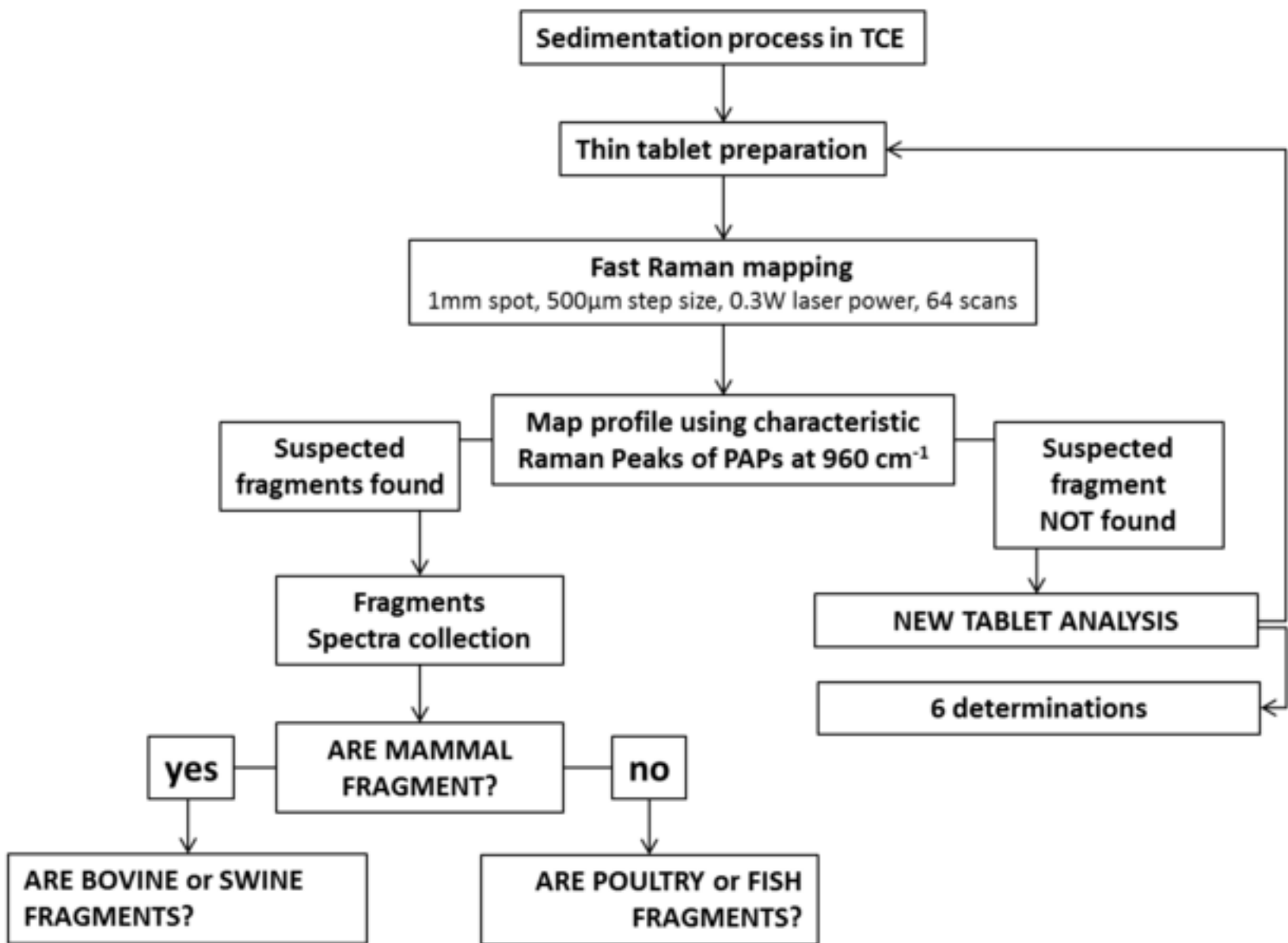


Figure 6
[Click here to download high resolution image](#)

