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Received: 19 January 2021 | Revised: 26 April 2021 | Accepted: 5 May 2021

DOI: 10.1002/jrs.6140

RESEARCH ARTICLE

Journal of RAMAN SPECTROSCOPY WILEY

Shifted excitation Raman difference spectroscopy as enabling technique for the analysis of animal feedstuff

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Funding information
Leibniz Science Campus Phosphorus Research Rostock

Abstract

To achieve the best performance and health in farm animals, high-quality pellets should be applied for feeding. Raw materials used for pellet production can have a significant influence on the nutritive and physical characteristics of the final product. A comprehensive quality control of raw materials and pellets is therefore essential. Optical inspection techniques show great promise as they enable fast, simple, and non-destructive analysis. This study demonstrates the potential of shifted excitation Raman difference spectroscopy (SERDS) for inspection of intact feed pellets and their constituents. SERDS combines the ability of conventional Raman spectroscopy to obtain chemically specific information from the sample with efficient fluorescence background rejection capabilities. The latter is an essential prerequisite for the application to highly fluorescent natural samples, for example, feedstuffs. A custom dual-wavelength diode laser with two slightly shifted emission wavelengths (785.2 and 784.6 nm) as required for SERDS is used as excitation light source. Results demonstrate that Raman signals can efficiently be separated from unwanted background contributions allowing for qualitative spatially resolved analysis of chicken feed pellets. Individual constituents present at levels down to 10 g/kg were successfully detected by means of their characteristic spectral signature. This highlights the large potential of SERDS and could pave the way for future inspection of raw materials and pellets at selected points along the process chain.

KEYWORDS

dual-wavelength diode laser, feed quality control, fluorescence rejection, non-destructive analysis, shifted excitation Raman difference spectroscopy

1 | INTRODUCTION

Supplying animals with feed in pelleted form can provide a number of advantages over mashed diets, including reduced dust formation and improved feed efficiency.[1,2] To fully exploit the benefits of pelleted diets and to achieve best animal performances due to breed and age-specific metabolic demands, it is very important that pellets of high nutritive and physical quality are used.[3,4] The pelletizing process in feed mills would ideally require a constant monitoring[5] to

identify potential issues already at an early stage. Conventional laboratory analytical methods[6] are not well suited for this purpose as they suffer from invasive and time-consuming analysis requiring complex and expensive instrumentation operated by experienced staff.

Optical techniques could provide a suitable alternative benefiting from fast and non-destructive analytical capabilities. As an example, near-infrared spectroscopy has a wide range of applications in the area of feed analysis,[7] for example, for the detection of melamine contaminations.[8,9] The technique has been applied in combination with microscopic imaging to assess the presence of meat and bone meal[10,11] and insect's meal[12] in selected feeds. Further studies report the classification of feed types[13] and feed ingredients.[14] Near-infrared spectroscopy was also applied to predict the chemical composition of compound feedstuffs for different types of animals[15,16] including the determination of proteins, fat and fiber.[17] Additionally, mid-infrared spectroscopy was used for the prediction of forage feed composition.[18]

Raman spectroscopy is another very promising technique that provides a chemically specific molecular fingerprint of the substance under investigation enabling qualitative but also quantitative analyses. One of the major issues of conventional Raman spectroscopy, preventing its widespread use with natural and biological samples, is strong fluorescence that can easily mask the characteristic Raman signals. In some cases, this can even prevent the acquisition of useful Raman spectra of animal feed.[19] A relatively common approach to address the fluorescence issue is Fourier-transform Raman spectroscopy using an excitation at 1064 nm. The technique has been used to predict digestibility of crude protein in feather meal of different origin[20] as well as for the detection of unwanted constituents in animal feed, for example, processed animal proteins,[21] melamine[22] and mycotoxins.[23] Another method to overcome fluorescence interference is the application of confocal Raman microscopy, for example, demonstrated for the determination of the colorant Sudan I in duck feed[24] or the detection of bacterial contamination in pelleted feedstuff.[25]

Shifted excitation Raman difference spectroscopy (SERDS) provides an efficient alternative solution to address fluorescence interference[26,27] and ambient daylight contributions.[28] Here, the excitation wavelength can be selected depending on the optical properties of the sample to circumvent undesired absorption and heating effects. Suitable light sources for SERDS have been reported from the short-wavelength visible at 457 nm up to the near-infrared spectral range at 830 nm.[29] The technique is based on the excitation of the sample with two laser emission lines slightly shifted in wavelength, for example, by approximately 0.6 nm at 785 nm excitation. This spectral distance of about 10 cm^{-1} has been selected to be in the range of the full-width at half-maximum of the Raman signals under investigation[27] and is suitable for the analysis of many solid and liquid specimens. While the Raman signals are shifted when the excitation wavelength is altered, fluorescence and other static contributions as ambient lights remain essentially unchanged. Subtracting both recorded Raman spectra therefore provides a neat way of separating the target Raman signals from those unwanted interfering contributions.[30] Due to recent developments in spectroscopic instrumentation, portable SERDS devices can now be deployed directly on site for in situ analysis, for example, demonstrated in an apple orchard.[31]

The purpose of the current study is to assess the potential of SERDS for the investigation of intact chicken feed pellets and corresponding raw materials used for pellet production. It will be shown that SERDS can efficiently recover the Raman spectroscopic signature of feed ingredients from fluorescence interference and that individual components can readily be distinguished from each other by means of their molecular fingerprint. Based on this initial assessment it will be demonstrated that it is possible to track the distribution of ingredients spatially resolved on intact chicken feed pellets.

2 | MATERIALS AND METHODS

2.1 | Experimental setup for SERDS

For our experiments, a compact laboratory setup for shifted excitation Raman difference spectroscopy was applied. A 785 nm dual-wavelength diode laser[32] was used as excitation light source and the emitted light was collimated by a lens with a focal length of 4.51 mm and a diameter of 6.33 mm (Thorlabs). After passing through a dual-stage optical isolator with 60 dB blocking (FI-780-5TVC, Qioptiq) a lens with a focal length of 13.86 mm and a diameter of 9.25 mm (Thorlabs) launches the laser light into an optical fiber cable with a core diameter of 100 μm (LEONI Fiber Optics). At the fiber output, the light is collimated by a lens with a focal length of 35 mm and a diameter of 25.4 mm (Thorlabs) and passes through two bandpass filters (LL01–785-25, Semrock) to remove amplified spontaneous emission. After reflection at a Raman long-pass filter (DI02-R785–25 × 36, Semrock) and a silver mirror (Qioptiq) the excitation light is focused downwards onto the sample by an achromatic lens with a focal length of 30 mm and a diameter of 25.4 mm (Thorlabs). The back-scattered light from the specimen is collected by the same lens in 180° geometry and reflected by the silver mirror. A set of three Raman long-pass filters (DI02-R785-25 × 36 and LP02-785RU-25, Semrock) reject the elastically scattered

radiation and anti-Stokes contributions while only the Raman Stokes scattered light (which is shifted to longer wavelengths with respect to the excitation laser light) passes through. By means of an achromatic lens with a focal length of 60 mm and a diameter of 25.4 mm (Qioptiq) the light is then launched into an optical fiber with a core diameter of 200 µm (Thorlabs) and transferred to the spectrometer having an optical resolution of 4 cm^{-1} (Tornado U1, Tornado Spectral Systems) with attached charge-coupled device detector (MityCCD H10141, CriticalLink) thermo-electrically cooled down to −10°C. The laser operation temperature of 25°C as well as the laser injection current was controlled by a custom laser driver (Toptica Photonics). In-house written software was used to set the laser operation parameters and to facilitate recording of the Raman spectra.

2.2 | Sample material and measurements

Initially, raw materials used for the preparation of the feed pellets were analyzed to obtain reference spectra of individual components. The homogeneous liquids soybean oil and methionine hydroxy analog were probed at five different spots each while for the solid components a number of 10 different spots each were investigated to account for potential sample heterogeneity. For all components 10 single Raman spectra at each of the two excitation wavelengths required for SERDS were recorded with integration times ranging from 0.5 to 5 s depending of the strength of fluorescence interference. An overview of the components as well as relevant spectral acquisition parameters is given in Table 1.

A total number of 10 randomly selected chicken starter feed pellets (diameter: 2 mm, length: 10 mm) were scanned along their long axis at 11 points each with a distance of 0.5 mm between the points, that is, covering a total length of 5 mm per pellet. For each spot 10 single Raman spectra for each of the two excitation wavelengths were recorded alternatively with integration times of 5 s. All investigations were conducted with an optical power of the excitation laser light at the sample of 20 mW.

The two Raman spectra recorded at the separate excitation wavelengths were normalized with respect to their intensity to compensate for potential background variations. The spectrum recorded at the first excitation wavelength of 785.2 nm (spectrum 1) was initially divided by the spectrum recorded at the second excitation wavelength of 784.6 nm (spectrum 2). Subsequently, the quotient (spectrum 1/spectrum 2) was fitted by a spline function (Matlab R2017a) to obtain a normalization function. Spectrum 2 was then multiplied by this normalization function (to adjust the baseline to that of spectrum 1) and the result was subtracted from spectrum 1. From the resulting difference spectrum the final SERDS spectrum was then computed using in-house written software based on a simple reconstruction algorithm.[33]

3 | RESULTS AND DISCUSSION

3.1 | Raman and SERDS spectra of feed components

The Raman spectra of an intact wheat grain recorded at the two laser emission wavelengths (784.6 and 785.2 nm) are presented in the top part of Figure 1 exhibiting strong

TABLE 1 Composition of chicken starter feed pellets and Raman acquisition parameters used for individual feed components

Ingredient	Content (g/kg)	Form of raw material	Number of spots probed	Integration time (s) for single spectrum
Soybean meal	420	Coarse powder	10	5
Wheat	291	Grain	10	5
Maize	200	Grain	10	5
Soybean oil	50	Liquid	5	5
Calcium carbonate	14.5	Fine powder	10	1
Premix	10	Coarse powder	10	1
Monocalcium phosphate	6.7	Coarse powder	10	1
Sodium chloride	3.2	Coarse powder	10	5
Methionine hydroxy analog	2.5	Liquid	5	0.5
Lysine sulfate	1.4	Coarse powder	10	0.5
Threonine	0.5	Fine powder	10	5

Note: The content (g/kg) values denote the amount of raw material for each ingredient used for pellet production.

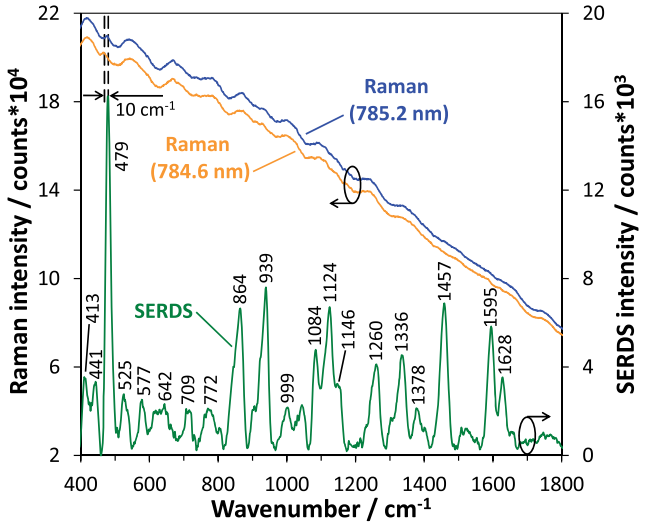

FIGURE 1 Raman spectra of an intact wheat grain excited at two slightly shifted wavelengths (blue and orange curve) and resulting SERDS spectrum (green curve) permitting for efficient separation of Raman signals from background interference. Characteristic Raman signals can be identified with the literature[36,37] [Colour figure can be viewed at wileyonlinelibrary.com]

fluorescence interference. Wheat is composed of different layers, namely the outer pericarp layer followed by the aleurone layer and finally the inner starchy endosperm of the seed. The observed high level of fluorescence originates from the pericarp and is in accordance with previous studies.[34] Due to this interference, most of the characteristic Raman signals expected from the aleurone layer (mainly ferulic acid) or the endosperm (starch granules embedded in a protein matrix)[35] are not readily observable. The strongest Raman signal of starch at 479 cm^{-1}, however, can be identified as its position is shifted between the blue and the orange spectrum as a result of the shift in excitation wavelength (see vertical dashed lines in Figure 1). Nevertheless, each of the two Raman spectra on their own would not be suitable for analytical purposes as strong fluorescence is masking the vast majority of the Raman spectroscopic information. After processing of the two Raman spectra as described in the previous section, a final SERDS spectrum (bottom green curve) is obtained. In this case, numerous characteristic Raman signals of different wheat constituents can effectively be separated from the interfering fluorescence contributions allowing for their identification with the literature.[36,37] As examples, prominent Raman bands of starch (864, 939, and 1457 cm^{-1}) and ferulic acid (1595 and 1628 cm^{-1}) are readily observable in the SERDS spectrum.

The other constituents used for the production of the feed pellets were investigated in a similar way and their obtained SERDS spectra are presented in Figure 2. All spectra give a unique fingerprint allowing for substance identification. For the major components with concentrations in the pellet of 10 g/kg or higher (see Table 1), specific target Raman bands for their identification within

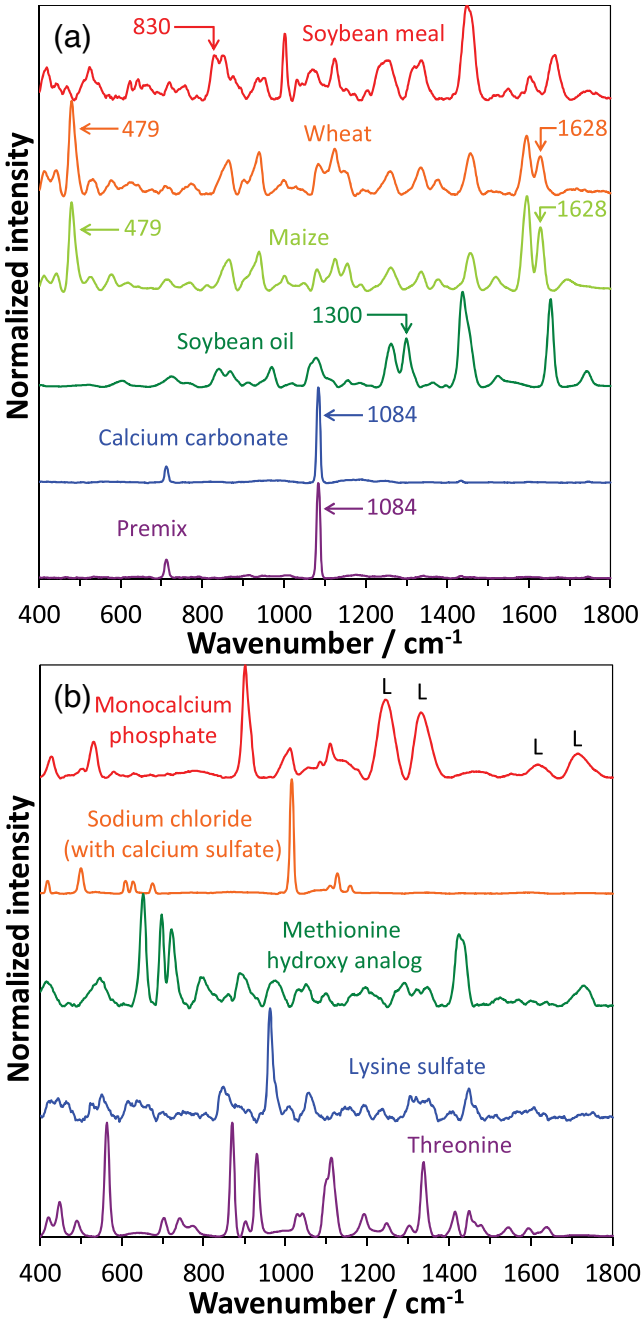

FIGURE 2 SERDS spectra of raw material from individual feed components with concentrations of 10 g/kg or higher (a) and lower than 10 g/kg (b) in final pellet (see Table 1). Target Raman bands for substance identification are indicated by arrows. Letter "L" denotes residual contributions from intense and spectrally narrow luminescent bands in Raman spectra of monocalcium phosphate not fully removed by SERDS. The spectra are vertically offset for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

the pellets, mainly the most intense Raman bands of individual substances, are indicated by arrows in Figure 2a. For soybean meal the medium intensity Raman band at 829 cm^{-1} has been selected as target band due to the fact that the (stronger) phenylalanine signal around 1000 cm^{-1} is also present in wheat and maize. In a similar way, the medium intensity band at 1300 cm^{-1} has been chosen as indicator for soybean oil as the strong C=C stretch lipid band at 1656 cm^{-1} is spectrally overlapping with the soybean meal amide I band at 1665 cm^{-1}. In the case of wheat and maize, two contributing substances were considered for further analysis, namely starch (Raman band at 479 cm^{-1}) and ferulic acid (Raman band at 1628 cm^{-1}).[34]

SERDS spectra could also be obtained for the minor components with concentrations in the pellet lower than 10 g/kg (see Figure 2b). Here, no target Raman bands are given as the detection of components in this concentration range is usually challenging without further techniques for Raman signal enhancement and this would be beyond the scope of the present study. In case of monocalcium phosphate residual contributions from intense and spectrally narrow luminescence bands are still present in the SERDS spectrum. Theoretically, all luminescence should be removed completely by SERDS as it does not follow the shift in the excitation wavelength. In the recorded Raman spectra, these luminescence bands are, however, more intense than the strongest Raman signal of monocalcium phosphate by a factor of up to 15. In contrast to broadband fluorescence interference, that is effectively rejected by SERDS as shown in Figure 1, the luminescence features have a much narrower spectral shape with full-widths at half-maximum as low as 34 cm^{-1} (i.e., less than 3 nm). Despite the powerful normalization procedure applied, this combination of high intensity and narrow bandwidth leads to inevitable baseline modulations in the difference spectrum between the two recorded Raman spectra ultimately causing the observed artifacts.

The reason for the incomplete removal of the luminescence bands in the SERDS spectrum is likely associated with luminescence bleaching. During laser illumination of the sample the luminescence intensity will slightly decrease over time but this reduction is not captured equally by spectra recorded at the two excitation wavelengths due to finite exposure times. A possible solution to overcome that issue would be the fast alternating acquisition of spectra at both wavelengths in the kilohertz range using a recently demonstrated charge-shifting concept.[38,39]

A comparison with SERDS spectra recorded on chemical-grade monocalcium phosphate (data not shown) confirms the spectral signature with the presence of all characteristic Raman bands but the above-mentioned luminescence bands are completely absent in this case. The cause of the luminescence features is thus likely associated with some (yet unknown) impurities in the food-grade monocalcium phosphate that are not present in its chemical-grade counterpart.

It is worth noting that for salt (sodium chloride) the recorded spectrum does not match the spectrum of pure sodium chloride which only exhibits characteristic Raman bands in the range below 400 cm^{-1}.[40] To resolve this issue one has to consider that natural rock salt (halite) resources often contain sulfate minerals as minor compounds.[41] In our case, by means of its characteristic Raman spectrum, the compound could be identified as anhydrite ($CaSO_4$).[42] For all 11 investigated feed pellet constituents the SERDS spectra permit for an efficient separation of the Raman bands from fluorescence interference and allowed for their identification with the literature (see Table 2).

Results show that SERDS can be applied irrespective of the physical state of the specimen and is equally well suited for whole grains, coarse or fine powders and liquids. The major advantage of the technique is that it can extract chemically specific information from the sample despite strong fluorescence interference, which is a common issue for the majority of biological and natural samples. As demonstrated in the case of sodium chloride the presence of minor components, that is, calcium sulfate, can readily be detected by means of the characteristic molecular fingerprint. Due to its ambient light rejection capabilities[28] the technique is suitable for in situ investigations outside usual laboratory environments.[31] In this way, SERDS as a non-destructive optical method can provide an efficient tool for identification and quality assessment of raw materials and byproducts, for example, binder materials, paving the way for inspection of components directly in feed processing plants.

3.2 | SERDS study of intact animal feed pellets

Following the initial Raman spectroscopic characterization of the individual constituents used for pellet manufacturing, spatially resolved SERDS measurements were conducted on intact whole feed pellets. The mean SERDS spectrum (black line) and the standard deviation (gray area) of 11 spectra recorded on one pellet along a length of 5 mm are exemplarily shown in Figure 3a. By means of the standard deviation it can be seen that pronounced variations in the content of starch, calcium carbonate and ferulic acid occur within the investigated area. Figure 3b visualizes the individual SERDS spectra

TABLE 2 Characteristic Raman band frequencies (in cm^{-1}) of investigated feed pellet components

Raw material	Raman band positions (cm^{-1})	References
Soybean meal	418, 443, 467, 523, 545, 622, 643, 656, 664, 719, 757, 830, 850, 875, 936, 952, **1002**, 1032, 1070, 1124, 1255, 1320, 1336, **1449**, 1603, 1665	Herrero et al.[43] and Jiang et al.[44]
Wheat	413, 441, **479**, 525, 577, 642, 709, 772, 864, **939**, 999, 1084, **1124**, 1146, 1260, 1336, 1378, **1457**, **1595**, 1628	Egging et al.[36] and Almeida et al.[37]
Maize	412, 442, **479**, 525, 577, 617, 713, 769, 811, 865, 939, 1001, 1081, 1125, 1155, 1187, 1262, 1336, 1378, 1457, 1519, **1596**, **1628**	Farber et al.[45] and Wellner et al.[46]
Soybean oil	603, 724, 840, 868, 970, 1078, 1156, **1262**, **1300**, 1365, **1438**, 1525, **1654**, 1742	El-Abassy et al.[47] and Sowoidnich and Kronfeldt[48]
Calcium carbonate, Premix	712, **1084**, 1434, 1754	Edwards et al.[49] and Wang et al.[50]
Monocalcium phosphate	428, 531, 580, **902**, 1011, 1110	Xu et al.[51] and Penel et al.[52]
Salt (NaCl + $CaSO_4$)[a]	418, 500, 609, 628, 675, **1015**, 1109, 1127, 1158	Berenblut et al.[42] and Culka and Jehlička[53]
Methionine hydroxy analog	416, 546, **652**, **697**, **722**, 793, 889, 975, 1032, 1051, 1099, 1196, 1290, 1320, 1347, **1425**, 1729	Grunenberg and Bougeard[54] and Lima et al.[55]
Lysine sulfate	444, 464, 551, 615, 640, 664, 847, **963**, 1008, 1056, 1306, 1319, 1351, 1448, 1608	Frushour and Koenig[56] and Verma and Goldner[57]
Threonine	421, 448, 490, **563**, 703, 741, 773, **870**, 903, **930**, 1029, 1042, **1101**, **1112**, 1193, 1248, 1303, **1338**, 1415, 1449, 1478, 1545, 1595, 1638	Krishnan et al.[58] and Pawlukojc et al.[59]

Note: Values given in bold indicate most intense Raman bands of each substance.

[a]Listed Raman bands belong only to anhydrite ($CaSO_4$) as rock salt (NaCl) does not exhibit Raman bands in the investigated spectral range.

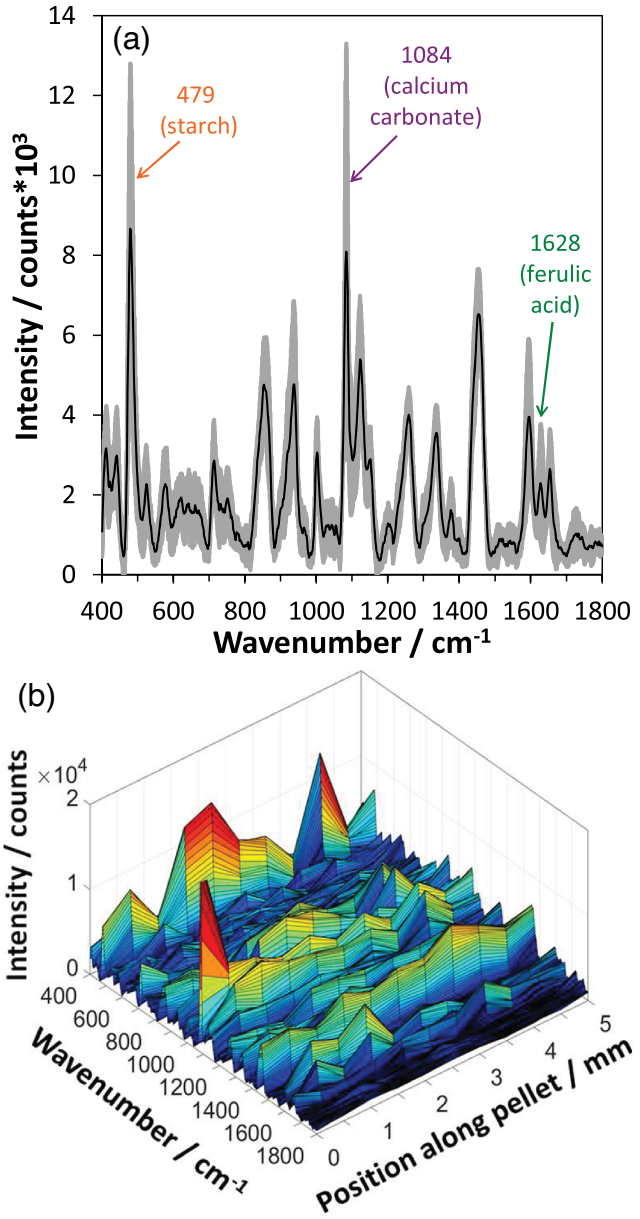

FIGURE 3 Mean (black solid line) and standard deviation (gray shaded area) of 11 SERDS spectra recorded from one single feed pellet. Contributions from starch, calcium carbonate, and ferulic acid show pronounced variations (a). 3D plot of SERDS spectra recorded at 11 positions with a distance of 0.5 mm between each other along the pellet over a total length of 5 mm highlights spatial variation of ingredients (b) [Colour figure can be viewed at wileyonlinelibrary.com]

recorded at different positions on the pellet to highlight the spatial distribution of individual components. Using the previously specified target Raman bands, it is now possible to track the presence of selected constituents along the pellet surface.

In Figure 4 the normalized intensities of the target Raman bands for soybean meal (830 cm^{-1}), starch (479 cm^{-1}), ferulic acid (1628 cm^{-1}), soybean oil (1300 cm^{-1}) and calcium carbonate (1084 cm^{-1}) are plotted in dependence of the spatial position along the pellets. As mentioned above, not always the most intense Raman band was selected but rather an intense band that is not spectrally overlapping with prominent Raman bands of the other ingredients. For all components a spatial variation within the probed area can be observed. Variations are most pronounced for soybean meal content in pellets 1, 6, and 7, for starch content in pellets 5 and 8, for ferulic acid content in pellets 7 and 8, for soybean oil content in pellets 2 and 3, and for calcium carbonate content in pellets 2 and 5.

From the data given in Figure 4 for the wheat and maize phases starch and ferulic acid exemplarily the three most intense spectra were identified and subsequently their average was calculated. The corresponding mean SERDS spectra (normalized to their respective maximum) are presented in Figure 5 together with vertical dashed lines indicating the target Raman bands for starch and ferulic acid. The most prominent Raman signals of the starch phase are located at 479 cm^{-1} and in the spectral range between 800 and 1500 cm^{-1}. For the ferulic acid phase, however, identifiable Raman signals can only be found above 1050 cm^{-1} with the two strongest signals appearing at 1595 and 1628 cm^{-1}. Hence, the two components contained in both wheat and maize can be readily separated by means of their distinct spectral patterns.

Our investigations have demonstrated that feed ingredients with concentrations above 10 g/kg can readily be identified within the processed pellet. In the applied configuration, the spatial distribution of these major pellet components can be determined using material-specific Raman bands and monitoring their intensity. Further improvements could likely be achieved using multivariate data analysis. Churchwell et al.[60] have recently shown that band-target entropy minimization can successfully be applied to recover individual component spectra from a complex multi-component matrix on condition that information about the target species is available or can be estimated. Applying this method on the SERDS spectra from feed pellets, it can be expected to identify ingredient composition and distribution even more reliably. As Raman signal intensity is proportional to the analyte concentration, a quantification of ingredients should, in principle, be possible as well. In our study, it has been shown that in wheat and maize it was possible to discriminate between the starch and ferulic acid phases based on their distinct spectral signatures. Coupling SERDS with multivariate analysis could thus potentially enable quantitative assessment of pellet composition, for example, to determine starch and ferulic acid content. In fact, dietary fiber is known to impair

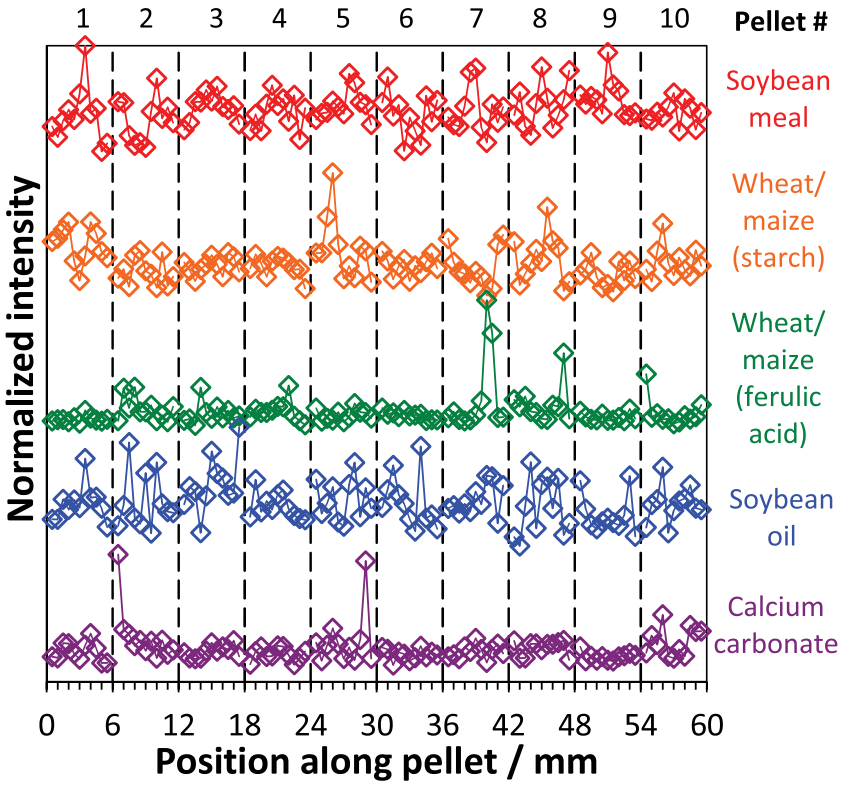

FIGURE 4 Normalized intensities of target Raman bands for soybean meal, starch, ferulic acid, soybean oil, and calcium carbonate highlight spatial variation of components along the pellets. Dashed vertical lines separate data obtained from different pellets. The curves are vertically offset for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

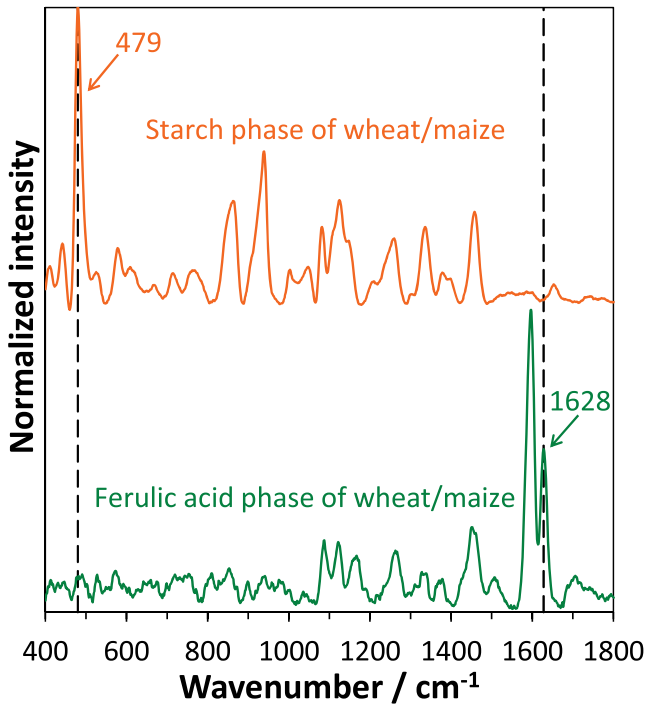

FIGURE 5 Mean SERDS spectra for starch and ferulic acid as selected phases present in wheat and maize. Data were calculated as the average of the three spectra with the highest intensity for the target Raman band (as indicated by arrows) for each component. The spectra are vertically offset for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

nutrient utilization in monogastric animals, while on the other hand, its beneficial effects on gut health, microbiota, and immunity are increasingly recognized.[61,62] As an example, the addition of 10 g/kg wheat bran to broiler feed reduced the level of cecal Salmonella shedding.[63] The established effect size in the current study thus meets the requirements of refining, for example, the starch-to-fiber ratio in monogastric feedstuff.

Minor components with concentrations below 10 g/kg within the pellet could be addressed as well, ideally if their Raman spectral signature is available from raw material analysis or known from the literature. For quantitative analysis of constituents at low concentration the heterogeneous distribution of the target substances needs to be considered. There exist several instrumental configurations to address this point, for example, applying an imaging technique by means of a multi-spot detection geometry.[64] A different approach has been presented by Griffen et al.[65] who used transmission Raman spectroscopy to obtain chemically specific information of large sampling volumes from solid samples in tablet form. In both cases, data analysis by means of multivariate statistics enabled to achieve detection limits of the respective target substances of less than 1 g/kg. This value corresponds to the concentration range of essential micronutrients such as minerals and vitamins in feed, which must be present in sufficient quantities to match metabolic demands.[66]

Overall, SERDS with its unique ability to extract chemically specific information from interfering contributions, for example, fluorescence or ambient light, coupled with appropriate hardware for representative sampling and advanced data evaluation methods has great potential for feedstuff analysis.

4 | CONCLUSIONS

The present study demonstrated SERDS as a promising analytical technique for feed pellet inspection and raw material identification. The chemically specific information obtained by means of SERDS enabled a qualitative assessment of the spatial distribution of ingredients with concentrations above 10 g/kg along the pellet. Our investigations form a solid basis for future studies to determine the potential of SERDS (ideally coupled with multivariate data analysis) as a valuable tool for quality control of raw materials and pellets at selected points along the process chain.

ACKNOWLEDGMENTS

This study was funded by the Leibniz Science Campus Phosphorus Research Rostock through the project "Shifted excitation Raman difference spectroscopy testing for analysis of inorganic phosphorus, inositol phosphates and myo-inositol in environmental and animal samples (SERAIP)". We would like to thank our industrial partner Trede & von Pein GmbH, Itzehoe, Germany for providing the raw materials and the feed pellets. We are grateful to Maria Krichler (Ferdinand-Braun-Institut gGmbH, Leibniz-Institut für Höchstfrequenztechnik) for developing the software to control the experimental setup.

CONFLICT OF INTEREST

None.

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How to cite this article: Sowoidnich K, Oster M, Wimmers K, Maiwald M, Sumpf B. Shifted excitation Raman difference spectroscopy as enabling technique for the analysis of animal feedstuff. *J Raman Spectrosc*. 2021;52:1418–1427. https://doi.org/10.1002/jrs.6140