Final report

AUSTRALIAN MEAT PROCESSOR CORPORATION

Heavy Metal Detection

Preliminary Investigation into the detection of heavy metals using Raman spectroscopy

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Contents

1.0 Executive summary

Due to the long half-life and potential to cause multiorgan failure and cancer, consumption of heavy metals poses a risk to human health. The livers of cattle accumulate heavy metals such as Cadmium through repeated exposure and poor excretion as it binds to protein thiol groups, which posing a risk to humans who consume offal. Consequently, acceptable limits of Cd have been established, however routine methods to determine if offal meets these limits is destructive, time intense, inefficient, requires trained personnel and is costly. In order to reduce wastage and financial loss while ensuring food safety, there is a need to develop additional screening methods which can be used routinely in abattoirs.

Vibrational spectroscopic methods such as Raman spectroscopy has distinct advantages as it is simple to operate, portable, rapid and requires no sample preparation. Although several studies have highlighted the potential for Raman spectroscopy to detect the biochemical changes in livers of fish and mice associated with Cd accumulation (Li et al., 2020; Hassan et al., 2024), as yet no research has been completed to assess the potential for use in beef abattoirs. Therefore, a study has been completed which aimed to investigate the potential for Raman spectroscopy to rapidly screen beef livers for the presence or absence of cadmium.

To this end, livers from 906 beef cattle including 267 from high-risk and 465 from medium risk postcodes which are removed from the supply chain were sampled as well as 174 livers from low-risk areas. Three replicate spectra were collected from the cut surface of livers of cattle using a Metrohm Tactic-ID 1074nm Raman device before a sample of liver was excised for Cd analysis using Inductively Coupled Plasma Mass Spectroscopy (ICP MS).

Of the livers measured, 114 livers from the high-risk samples and 26 of the livers sampled in the medium risk category had concentration of Cd concentration greater than 0. The mean Cd was 0.009mg/kg (s.d = 0.04) in livers from medium-risk areas and 0.23mg/kg (s.d = 0.39) for livers in high-risk areas, while the maximum concentrations recorded were 0.39mg/kg and 2.29mg/kg for livers from medium risk and high-risk areas respectively.

Chemometric modelling was able to classify livers based on risk category with errors of between 12 – 28%, however models to classify samples into low (0 – 0.1 mg/kg), medium (0.1 – 1 mg/kg) and high (>1mg/kg) groups based on their Cd concentrations yielded poor results with false negative rates (FNR) as high as 45% and a large number of samples were classified as unassigned. Similarly, spectra were unable to predict the Cd concentration resulting in an R^2 of 0.33, an RMSE of 0.20 and an RPD of 1.22.

Despite the poor predictive models, spectra from livers which have higher concentrations of cadmium also have a higher fluorescence when compared to livers with lower levels. Furthermore, spectral features at 1270 cm $^{-1}$, 1300 cm $^{-1}$, 1350 cm $^{-1}$, 1450 cm $^{-1}$, 1598 cm $^{-1}$ and 1650 cm $^{-1}$ varied between cadmium classes. This was consistent with previous research which suggested an increase in collagen due to fibrosis of the liver and increases in lipid peroxidation could be observed in spectra (Li et al., 2020). However, as ageing and infections can also cause fibrosis and oxidative stress (Schmucker, 2005), it remains unclear whether the changes noted were a reflection of the risk category older animals are more susceptible to increased accumulation and are more likely to have liver damage or Cd accumulation given the incidence of Cd was low. Therefore, further research is warranted using a targeted population to increase the incidence in Cd found.

2.0 Introduction

Heavy metals are elements which can pose a risk to human health as they have long biological half lives and as a result do not degrade in the environment or biological tissues causing accumulation (Salim et al., 2023). Regardless of its source, contamination due to heavy metal pollution has raised public concern regarding both long and short term environmental and health effects as consumption of elements such as cadmium (Cd) potential to cause multi organ failure and cancer (Tchounwou, Yedjou, Patlolla & Sutton, 2012).

With a combination of dietary exposure and poor excretion abilities, livestock including sheep and cattle are prone to accumulation of heavy metals in the kidneys and liver where they bind to free protein-thiol groups posing a risk to humans who consume the offal (Pompe-Gotal & Crnic, 2002). Therefore, acceptable limits of Cd have been established, however these vary by region and country (Lucas & MacLachlan, 2020). Currently, the National Residue Scheme is responsible for assuring that beef carcasses are within acceptable limits for heavy metals. To achieve this, carcases are tested annually based on a risk assessment using laboratory-based techniques including atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS). However, these methods are destructive, time intense, inefficient, require trained personnel and are costly. Consequently, only ~ 300 carcases are tested annually suggesting the potential for error, oversight and reputational risk. Subsequently, the offal from at risk carcases is removed from the supply chain. In order to reduce wastage and financial loss while ensuring food safety, there is a need to develop additional screening methods which can be used routinely in abattoirs.

Vibrational spectroscopic methods have distinct advantages as they are simple to operate, portable, rapid and require no sample preparation. Raman spectroscopy is one such non-destructive rapid spectroscopic technique which utilises the interaction between light and the chemical bonds of matter to provide information on the structure, composition, and molecular interactions of matter, effectively providing a “chemical fingerprint” (Das & Agrawal, 2011). Although Raman spectroscopy has been overlooked for heavy metal detection in muscle foods as it is unable to directly detect heavy metal ions, there is an increasing number of studies which demonstrate Raman spectroscopy is able to quantify heavy metal concentrations through the biochemical changes that are associated with accumulation, allowing heavy metals to be recognised in spectra (He et al., 2022).

Indeed, studies which have exposed mice to Cd showed changes in liver cells including a reduction in the bands 748cm^{-1}, 1082cm^{-1}, 1128cm^{-1} and 1585cm^{-1} which are associated with cytochrome C and increases in the bands associated with collagen suggesting fibrosis in the liver tissues of Cd affected mice (Li et al., 2020). Further damage to the liver as a result of Cd accumulation was also observed in mice suggesting changes to the DNA proteins peroxidation of lipids and cell apoptosis and necrosis were also evident in spectral changes of livers from Cd affected mice (Li et al., 2020). Similar studies in which fish samples were spiked with increasing levels of Cd have suggested similar results stating concentrations of peaks between 300 – 700 cm^{-1} were altered by increasing Cd content, particularly peaks at 307cm^{-1} and 719cm^{-1} (Hassan et al., 2024).

Given this and the sensitivity shown by the technique, it is proposed to use Raman spectroscopy to rapidly screen beef livers for the presence or absence of heavy metals, enabling industry to safeguard their interests with robust and widespread assessment for heavy metals. In practice, this approach would fit at offal removal stage of beef carcass fabrication. Furthermore, a screening tool based on Raman spectroscopy will reduce the cost and time associated with current method used for chemical screening. It will allow more offal to be sold with an assurance to consumers that it meets their expectation – namely, beef that is “safe”, free of residues and heavy metals.

3.0 Project objectives

The objective of the project was to investigate the potential for a hand-held Raman device to detect heavy metal contamination of offal.

4.0 Methodology

Pilot Study

As spectroscopic parameters are currently not known for offal, an initial in- plant trial was conducted to ascertain the appropriate laser power and integration time to collect quality spectra on offal and the location for measurement. To this end, Raman spectroscopy measurements were taken on a liver, in various positions with numerous equipment parameters once the offal has been collected post-mortem in the packaging room, immediately after harvesting. A comparison of the quality of the spectra was completed and the optimal parameters and best position was determined to be on the cut surface of the liver.

Trial Methodology

Livers from 906 beef cattle including 267 from high-risk and 465 from medium risk postcodes which are removed from the supply chain were sampled as well as 174 livers from low-risk areas. Three replicate spectra were collected from the cut surface of livers of cattle produced across Australia using a Metrohm Tactic-ID 1074nm Raman device with a 90% laser power and 10 s interval. Once spectra were collected a ~30g sample of liver was excised, freeze dried and ground for further testing using Inductively Coupled Plasma Mass Spectroscopy (ICP MS) (Carrilho, Gonzalez, Nogueira, Cruz & Nóbrega, 2002) to determine the concentration of heavy metals, namely cadmium.

Partial Least Squares Discriminant Analysis (PLS-DA) and XGBoost were then undertaken to determine the potential for the Raman spectra to classify samples based on the concentrations given by ICP MS. Further modelling including Partial Least Squares and Principal Component Analysis were also trialled. Analysis was completed using Matlab (The Mathworks Inc., 2013) and R Core Software (R Core Team, 2023) using "*Chemospec*" (Hanson, 2020), "*mdatools" (Kucheryavskiy, 2020) and "pls" (Mevik, Wehrens & Liland, 2011)* packages. Predictive outcomes for PLS regression models were reported as the squared correlation between the predicted and observed values (R^2), the root mean squared error (RMSE), the root mean squared error of the cross validation (RMSECV), the bias and the (RPD)

5.0 Project outcomes

Of the livers measured, 114 livers from the high-risk samples yielded a concentration of Cd concentration greater than 0 while 26 of the livers sampled in the medium risk category yielded a concentration of greater than 0. The mean concentration measured in livers from low-risk areas was 0, 0.009mg/kg (s.d = 0.04) in livers from medium-risk areas and 0.23mg/kg (s.d = 0.39) for livers in high-risk areas, while the maximum concentrations recorded were 0.39mg/kg and 2.29mg/kg for livers from medium risk and high-risk areas respectively.

When the concentrations found are compared to the maximum allowable concentrations (Table 1), 11% of livers were over the maximum allowed for Singapore, 8.3% were over the allowable concentrations for the Eurasian Economic Union, 5.1% were over the allowable concentrations for the EU. With the highest allowable maximum levels, only 1.8 and 1.2% of livers were over the allowable concentrations for Malaysia and Australia respectively.

Table 1. Cadmium concentrations measured in livers from low, medium and high-risk cattle

Country/Region (Maximum Level mg/kg)	Livers which exceeded these levels	Percentage of Total Measured
Singapore (0.2)	100	11
Eurasian Economic Union (0.3)	75	8.3
EU (0.5)	46	5.1
Malaysia (1)	16	1.8
Australia (1.25)	11	1.2

As 57% of the high samples yielded a concentration of cadmium and samples from each category were collected together, initial analysis has been completed to ascertain the ability of Raman spectra to classify the livers based on their risk assessment. PLS-DA models were capable of discriminating unknown samples into a risk class with errors ranging from 12 – 28% (Table 2 and 3) after being split randomly into calibration and validation test sets.

Table 2. Confusion Matrix for the calibration model to classify livers into risk categories based on Raman spectroscopy.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
High	0.61176	0.13436	0.86564	0.38824	170	0.20353	0.63030	0.62090
Low	0.89764	0.08652	0.91348	0.10236	127	0.08974	0.72611	0.80282
Medium	0.74006	0.20202	0.79798	0.25994	327	0.23237	0.80132	0.76948

Table 3. Confusion Matrix for the validation model to classify livers into risk categories based on Raman spectroscopy.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
High	0.48837	0.13260	0.86740	0.51163	86	0.25468	0.63636	0.55263
Low	0.74468	0.09545	0.90455	0.25532	47	0.12360	0.62500	0.67961
Medium	0.76119	0.32331	0.67669	0.23881	134	0.28090	0.70345	0.73118

This resulted in 43 livers correctly assigned to the high-risk category (Table 4). While this demonstrates that there are spectral differences between livers from each risk category, some high and medium risk livers were incorrectly assigned.

Table 4. Confusion Matrix for the validation model to classify livers into risk categories based on Raman spectroscopy.

	Actual Class

	High	Low	Medium
Predicted as High	43	3	21
Predicted as Low	10	35	11
Predicted as Medium	34	9	102
Predicted as Unassigned	0	0	0

Further modelling has been completed to ascertain the value of non-linear models which can be prone to overfitting in calibration models. While this was evident in the results from the calibration data, validation data showed XGBoost discriminate analysis (XGB-DA) a non-linear model provided a realistic performance on the test set with errors of 7 – 22% (Tables 5 and 6).

Table 5. Confusion matrix for the calibration model for the classification of risk using XGBoost Discrimination Analysis (XGB-DA).

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
High	1.00	0.00	1.00	0.00	170	0.00	1.00	1.00
Low	1.00	0.00	1.00	0.00	127	0.00	1.00	1.00
Medium	1.00	0.00	1.00	0.00	327	0.00	1.00	1.00

Table 6. Confusion matrix for the validation model for the classification of risk using XGBoost Discrimination Analysis (XGB-DA).

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
High	0.54651	0.07735	0.92265	0.45349	86	0.19850	0.77049	0.63946
Low	0.70213	0.01818	0.98182	0.29787	47	0.06742	0.89189	0.78571
Medium	0.91045	0.35338	0.64662	0.08955	134	0.22097	0.72189	0.80528

As shown in Table 7, these models accurately predicted the class of 202 livers.

Table 7. Confusion table for the validation model for the classification of risk using XGBoost Discrimination Analysis (XGB-DA).

	Actual Class		
	High	Low	Medium
Predicted as High	47	3	11
Predicted as Low	3	33	1
Predicted as Medium	36	11	122

Predicted as Unassigned	0	0	0

When samples were randomly selected from low (0 – 0.1 mg/kg), medium (0.1 – 1 mg/kg) and high (>1mg/kg) classes based on their Cd concentrations and not risk category to create even groups only 16 samples from each group were used. Predictive outcomes from this model are poor with FNR as high as 45% and low F1 statistics (Table 8) and furthermore a large number of samples were identified as unassigned (Table 9).

Table 8. Confusion matrix for the calibration model for the prediction of cadmium based on export threshold.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
High	0.54651	0.07735	0.92265	0.45349	86	0.19850	0.77049	0.63946
Low	0.70213	0.01818	0.98182	0.29787	47	0.06742	0.89189	0.78571
Medium	0.91045	0.35338	0.64662	0.08955	134	0.22097	0.72189	0.80528

Table 8. Confusion table for the validation model for the classification of cadmium concentration using Partial Least Square Discrimination Analysis (PLS-DA).

	Actual Class		
	High	Low	Medium
Predicted as High	3	1	2
Predicted as Low	2	6	2
Predicted as Medium	0	0	0
Predicted as Unassigned	11	9	12

PLS regression analysis to predict the Cd concentrations yielded an R^2 of 0.33, an RMSE of 0.20 and an RPD of 1.22 (Fig 1). However, cross validation of this model reduces the predictive outcomes to an R^2_{cv} of 0.16, an RMSE of 0.22, and an RPD of 1.09 suggesting the model is not robust.

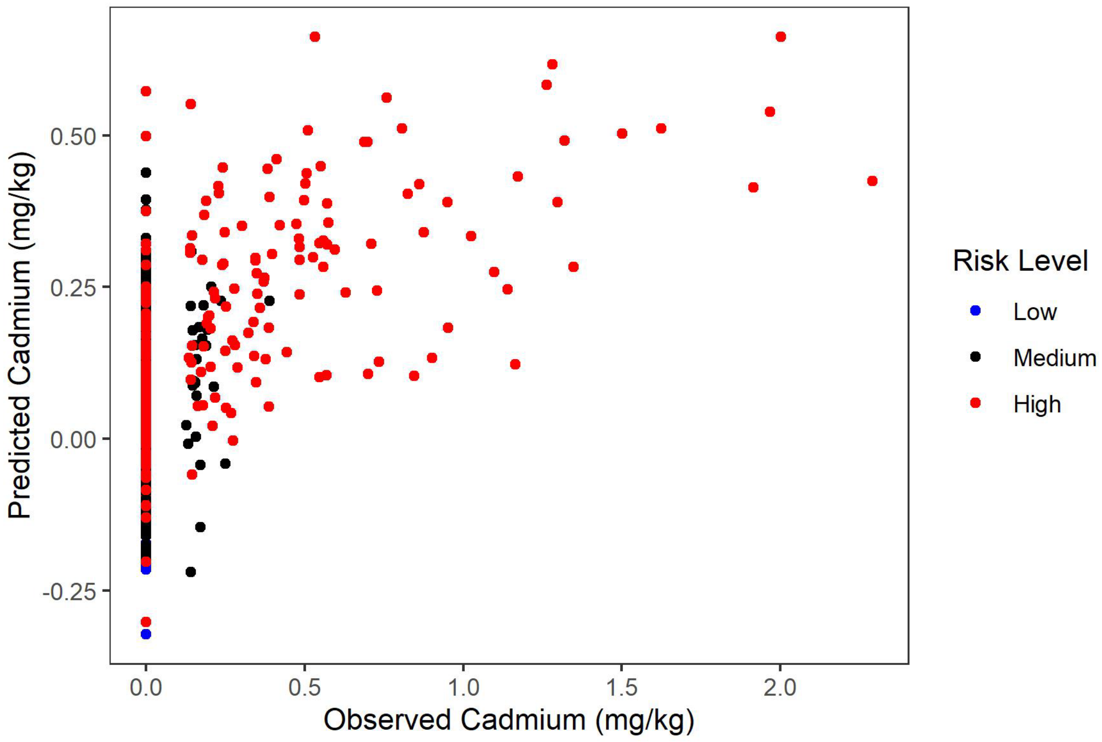

Figure 1. PLS prediction of Cadmium concentrations using the whole data set.

When the numbers of samples not containing cadmium are reduced in models to balance out the population, the model uses only 48 samples and the non-cadmium containing samples have a bigger influence on model outcomes. Consequently, the predictive outcomes are poorer resulting in an R^2 of 0.096, an RMSE of 0.639 and an RPD of 1.06 despite the plot of predicted vs observed cadmium concentrations suggesting a relationship is present (Fig 2).

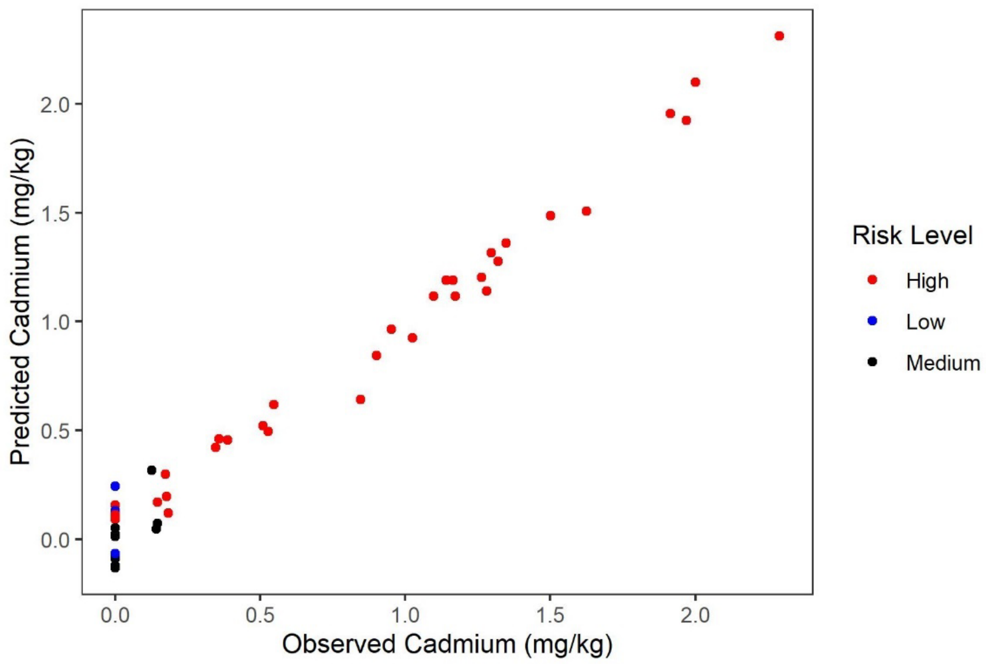

Figure 2. PLS prediction of Cadmium concentrations using a subset of data with no cadmium present.

When the average spectra from each of the classes of cadmium concentrations are considered (Fig 3), it is evident that spectra from livers which have higher concentrations of cadmium also have a higher fluorescence when compared to livers with lower levels.

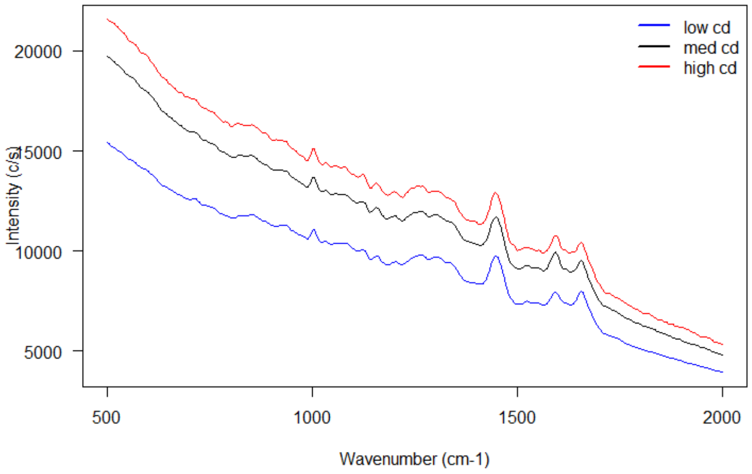

Figure 3. Average raw spectra based on cadmium classes where low represents 0 mg/kg cadmium, medium represents 0.1 to 1 mg/kg cadmium and high represents over 1 mg/kgs.

As shown in Fig 4, baseline correction to remove the fluorescence demonstrated spectral features vary between cadmium classes at 1270 cm^{-1}, 1300 cm^{-1}, 1350 cm^{-1}, 1450 cm^{-1}, 1598 cm^{-1} and 1650 cm^{-1}.

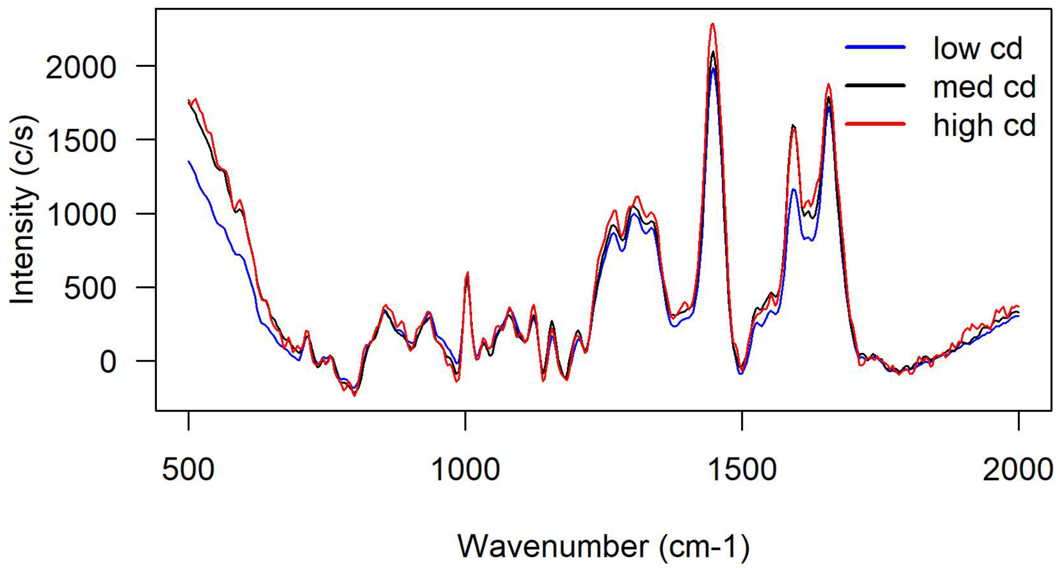

Figure 4. Average baseline corrected spectra based on cadmium classes where low represents 0 mg/kg cadmium, medium represents 0.1 to 1 mg/kg cadmium and high represents over 1 mg/kgs.

6.0 Discussion

Overall data suggests the overall incidence of cadmium in beef livers is lower than reported by the NRS as only 11% of samples collected yielded Cd concentrations greater than the limit of reporting (LOR), while the NRS 2022 – 2023 beef residue testing dataset suggests that 77% of livers measured had Cd concentrations greater than the LOR (Department of Agriculture, 2024). Consequently, the incidence of Cd was not high enough to create robust calibration models.

This is confirmed by both PLS-DA models which were unable to correctly classify samples or predict the concentration of Cd present. While randomly selecting samples to balance classes was trialled, this resulted in a small calibration model with only 16 samples in each group. Consequently, models lacked spectral information on the biochemical changes which are associated with the presence of heavy metals. Therefore, models were unable to distinguish between liver samples which contained higher levels of Cd and those without resulting in a high proportion of samples designated as unassigned.

Likewise, the small proportion of samples affected also reduced the ability of PLS models to quantitatively predict Cd concentrations resulting in an R^2 of 0.33 and an R^2_{cv} of 0.16. However, spectra were able to detect differences in livers based on risk category with models suggesting it was possible to discriminate between classes with errors ranging between 12 – 28%. While this demonstrates that the biochemical differences associated with livers from each risk category are able to be detected by Raman spectra, the incorrect assignment of some high and medium risk livers occurred. Although this increases the error to levels which may be unacceptable for application, this may be promising given not all livers in the medium or high-risk categories had Cd present as 26 livers from the medium risk category has cadmium present, while 153 livers from the high-risk category did not have cadmium present.

Such predictive outcomes are significantly lower than previous research which has been completed on fish, which suggests several Raman spectral peaks can detect Cd in fish samples with accuracies of up to $R^2 = 0.99$ (Hassan et al., 2024). However, a comparison between such studies is limited given that Hassan et al. (2024) directly contaminated fish, spiking *Tilapia niloticus* samples with prepared stock solutions with 0.005, 0.01, 0.05, 1.0 and 5.0ppm of Cd and therefore the heavy metals did not undergo the same metabolic processes which would have occurred when cattle ingested the Cd. It is also unclear whether differences in samples analysed account for some of the decrease in accuracy given that Hassan et al. (2024) measured fish muscle while the current study has analysed liver given it is higher in haem.

Although they have not quantified or classified Cd concentrations, Raman studies which demonstrate changes to the spectra with increasing Cd concentrations in mice livers have suggested increased Cd concentrations resulted in less resolved peaks and less signal to noise ratio (Li et al., 2020). This agrees with the current study which found fluorescence increased and peaks were less resolved when the Cd concentrations increased. This may be due to the destruction of cells with Cd exposure resulting in degenerated and disorderly cells (Li et al., 2020), which may alter the path of the excitation energy when compared to livers not affected by Cd accumulation.

Further spectral changes were also consistent with previous research as spectral features vary between cadmium classes with increases at 1270 cm^{-1}, 1300 cm^{-1}, 1350 cm^{-1}, 1450 cm^{-1}, 1598 cm^{-1} and 1650 cm^{-1}. Li et al. (2020) have attributed similar spectral changes to the CH_2 bending of proteins and phospholipids (1446 – 1448 cm^{-1}), the C=C bending of Cytochrome c, Phenylalanine and hydroxyproline protein (1585 cm^{-1}) and the Amide I C == O stretching of collagen and fatty acids (1657 cm^{-1}) which occur with the damage done to livers as a result of the presence of Cd. Primary damage was evident in spectra through changes to haem proteins such as a reduction Cytochrome C, increases in lipid peroxidation which leads to changes to the transparency of cells and increases in collagen reflecting fibrosis of liver tissues as well as cell death due to oxidative stress in cells which occur due to the accumulation of Cd (Li et al., 2020).

Given ruminants are born with a low concentration of heavy metals which accumulates over their lifespan due to the long half-lives and poor excretion rates of heavy metals, mature animals have demonstrated increased concentrations of Cd (Zenad et al., 2020). While accumulation of Cd causes the biochemical changes to the liver, ageing also causes increased oxidative stress, cell dysfunction and structural metamorphoses of liver cells (Schmucker, 2005). Furthermore, fibrosis of the liver can also be triggered when liver damage is sustained either through disease such as hepatic lipidosis or infections such as echinococcosis (Zhang et al., 2023). Therefore, it is hypothesised part of the error and low prediction accuracies can be attributed to spectra differentiating livers based on similar morphology due to age or liver damage from alternate causes rather than damage which can be attributed to an increased Cd concentration. This may explain why it was possible to accurately predict risk class, given risk classes are based on age and location, yet further analysis to consider the ability to predict the incidence of Cd within each class is not possible due to the low incidence within the medium and high-risk categories.

7.0 Conclusions / recommendations

While preliminary spectral analysis suggests that Raman spectra are able to detect differences between livers based on the overall fluorescence and peaks related to increased fibrosis and cell death, the incidence of Cd was not great enough to create robust calibration models. Further research is required with a larger population of high risk samples and more specific regional targeting of high-risk animals.

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