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Effective Detection of Proteins Following Electrophoresis: Using Extracts of Locally Available Food Species

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ABSTRACT

Procedures in life sciences research laboratories often require chemicals and plasticware that are costly, toxic or pose a risk to the environment. Therefore, sustainable alternatives would be of interest, provided that they generate suitable data quality. Coomassie blue and silver staining are the most widely used methods for detecting proteins following electrophoresis in the laboratory. However, their use presents challenges in terms of safety and waste management. In the current study, aqueous extracts were prepared from a series of common food species and evaluated as alternative stains for protein detection. Beets, blueberries, purple cabbage, raspberries and strawberries were employed to stain identical proteins separated under the same conditions in electrophoresis gels. Extracts of the first two species resulted in protein bands that were detectable through visible light transillumination, whereas extracts from all five species generated specific protein bands under ultraviolet light. The raspberry-derived extract was selected for further study based on the brightness of the fluorescent protein bands and minimal background staining. For both bovine serum albumin and lysozyme at 2.5 µg and 0.5 µg protein per band, the mean signal intensities obtained with raspberry extract staining were just below half of those obtained with Coomassie blue. Furthermore, the mean intensities using raspberry extract were equivalent to those obtained using Coomassie blue in the detection of 0.1 µg protein. Therefore, raspberry could be used to produce an effective stain for the routine laboratory analysis of proteins.

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Introduction

Laboratory research in the life sciences relies on a variety of techniques that require specialized equipment and reagents. A common method for the efficient separation and analysis of proteins is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, proteins are unfolded by heating and by interacting with SDS, which also imparts a uniform negative charge. An electric field then causes protein movement through the gel system, with proteins first concentrated in a stacking gel and then migrating according to their chain lengths by sieving through the polyacrylamide matrix of the separating gel (Srinivas, 2012). SDS-PAGE is highly informative, as proteins can be directly separated based on molecular weight, detected by staining and quantified by densitometry of the stained bands. It is also versatile, as the separated proteins can be excised for mass spectrometric analysis or transferred from the gel to a membrane for further investigation using antibodies.

Following SDS-PAGE, the separated proteins are typically detected by staining with Coomassie blue (Chevalier, 2010; Smejkal, 2004; Steinberg, 2009; Sundaram et al., 2012). Through visible light imaging, this dye provides good sensitivity, low background and wide-ranging protein recognition. Other commonly used options for detection with visible light include multi-step silver staining procedures (Smejkal, 2004; Steinberg, 2009; Sundaram et al., 2012; Weiss et al., 2009) and negative protein detection methods employing copper, zinc or cobalt ions (Dzandu et al., 1988; Lee et al., 1987). Fluorescent or luminescent stains such as the SYPRO™ compounds are also effective (Steinberg, 2009; Steinberg et al., 1996). Silver and fluorescent stains are more sensitive than Coomassie blue, allowing nanogram levels of a protein to be detected (Smejkal, 2004; Steinberg, 2009; Weiss et al., 2009). In addition, the metal ion-based negative staining is rapid and reversible (Sundaram et al., 2012). Nonetheless, all of these methods have drawbacks. For example, silver staining typically requires the use of toxic compounds such as formaldehyde and glutaraldehyde (Smejkal, 2004, Weiss et al., 2009), which necessitate extensive precautions and waste disposal considerations. Metal ion-based staining also requires precautions and particular disposal of the copper, cobalt or zinc solutions used. In addition, negative staining results in an opaque gel with clear protein bands, thereby precluding quantitation of the protein present. Furthermore, most fluorescent stains are costly and contain proprietary reagents. Coomassie blue staining involves the use of one of two commercially available staining reagents (Coomassie blue R- or G-250) and moderate volumes of methanol or isopropanol to dissolve the stain and to destain the gel. Isopropanol and methanol are known to present multiple hazards and Coomassie blue has been reported to be toxic (Hoffman and Guz, 1961). To mitigate the toxicity of Coomassie blue and adhere to the safety and disposal requirements of the solvent, an alternative staining method was developed that employed Kimwipe™ tissues (Dorri & Kurien, 2010). The method removed the diffused dye for safer disposal and reduced solvent use (Dorri & Kurien, 2010). Nevertheless, toxicity, procurement and transportation costs were reduced rather than eliminated by that approach.

Fruits and vegetables contain a variety of pigments, some of which may offer protein staining options that circumvent the expense, safety and environmental issues that can arise with the use of common laboratory stains. For example, blackberry (*Rubus fruticosus*) anthocyanin was used to stain cell nuclei when there was a shortage of haematoxylin in the 1970s (Al-Tikritti & Walker, 1978). Additionally, beet extracts were found to be informative stains in mammalian tissue histology (Udonkang et al., 2018).

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A few plant extracts and natural products have also been evaluated as stains for use with SDS-PAGE. Tannic acid in a concentrated acetate buffer stained proteins following electrophoresis, resulting in white bands (Aoki et al., 1981). Henna (*Lawsonia inermis*) was also investigated as a protein stain (Ali & Sayeed, 1990). Turmeric (*Curcuma longa*) and the component curcumin were shown to stain proteins in gels, resulting in fluorescent bands under ultraviolet light (Kurien et al., 2012). More recently, stains from green and brown walnut (*Juglans regia*) husks were found to rapidly reveal bands under visible light following protein electrophoresis (Mushtaq et al., 2021). Although solvents such as methanol were used in some cases – as fixatives or for other purposes – the actual staining solutions were inexpensive and generated no toxic waste.

An ideal and sustainable protein stain for routine use after SDS-PAGE would be local, readily available, low-cost, non-toxic, and effective in protein detection. Several plant species produced commercially for food met many of these criteria. However, their utility in staining proteins remained to be evaluated. The hypothesis was that one or more locally grown species would generate an effective protein stain for use following SDS-PAGE. Therefore, the goals of this study were to prepare extracts from several highly pigmented food species that are grown in the Maritime provinces of Canada and to determine their effectiveness in protein detection.

Methods

Preparation of Food Species Extracts:

For the screening experiments, raspberries (*Rubus idaeus*), strawberries (*Fragaria* x *ananassa*), blueberries (*Vaccinium angustifolium*), beets (*Beta vulgaris)* and purple cabbage (*Brassica oleracea*, red variety) were purchased from grocery stores and local markets in September and October, 2020. The produce used in this study was cultivated in a variety of locations. However, each species was chosen because it is also grown locally as a commercial crop.

For the initial qualitative experiments, six aqueous extracts were prepared from each species. Since the Dalhousie University campus was closed to students in the fall of 2020, each student prepared an extract at home from fresh or frozen produce. The following precautions were taken during extract preparation: the kitchen area was wiped clean, hands were thoroughly washed and all plastic and glassware items were treated with boiling water prior to use. These measures were taken to avoid microbial growth in the prepared extracts. An amount of produce equivalent to at least 300 mL was thawed (if frozen) and then broken by hand (if soft) or by chopping, cutting or mechanically separating into small pieces in preparation for extraction (Fig 1A). A 250-mL volume of the prepared produce was placed in a pot with an equal volume of water and brought to a boil on a kitchen stove. It was then allowed to boil very gently (simmer) while remaining uncovered for 30 minutes and then removed from the burner and allowed to cool for 1 hour (Fig 1B). The cooled liquid was separated from insoluble materials by filtration through a coffee filter placed in a coffee-making funnel or a similar apparatus and set up over an empty container to collect the filtrate (Fig 1C). The latter was then placed in a leak-proof container and either delivered following COVID-19 safety measures or sent by mail to the Department of Biochemistry & Molecular Biology at Dalhousie University. Upon receipt, all aqueous extracts were stored in a departmental cold room set to 6-8 ºC until use.

For the quantitative experiments, raspberry extracts were prepared precisely as above, except in the laboratory setting. The raspberries used were a 1:1

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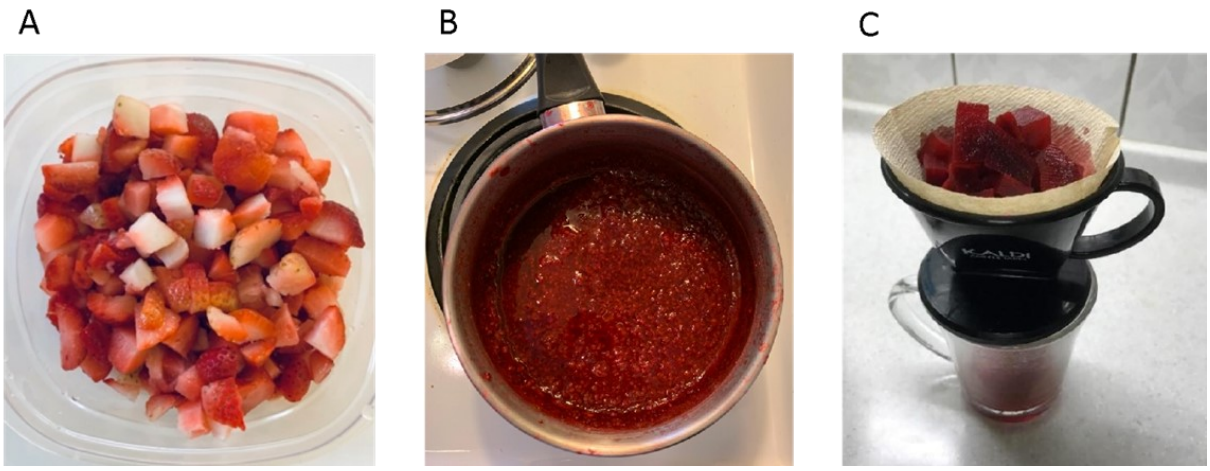

Figure 1 Representative images showing the preparation of aqueous extracts from locally available produce species. Photos were taken by students in their homes. Panel A shows strawberries (*Fragaria x ananassa*) after chopping, panel B shows raspberries (*R. idaeus*) after boiling and panel C shows beets (*B. vulgaris*) during filtration.

mixture of frozen fruit from Serbia (President's Choice, Superstore) and Chile (Great Value, Walmart). The filter used was an unbleached basket coffee filter (President's Choice, Superstore).

SDS-PAGE of Proteins:

SDS-PAGE (Laemmli, 1970) was conducted using a Mini-Protein II gel apparatus and power supply (Bio-Rad) following the manufacturer's instructions. Sets of 15-well gels were prepared, each consisting of a 12% acrylamide separating gel with a 3% acrylamide stacking gel. The gel buffers, sample buffer, ß-mercaptoethanol (all Bio-Rad) and acrylamide solution (Fisher) were used as directed by the manufacturers. The Tris-glycine-SDS running buffer was also prepared according to the instructions provided by Bio-Rad. Proteins used included 10-175 kDa pre-stained protein markers (Bio-Shop), Precision Plus Protein unstained markers (Bio-Rad), bovine serum albumin (BSA, Sigma-Aldrich), and hen egg white lysozyme (Worthington Biochemical Corporation). Blood plasma was obtained from rats maintained in accordance with the guidelines of the Canadian Council on Animal Care.

Proteins were combined with a reducing sample buffer and boiled for 5 minutes, centrifuged for a few seconds and loaded onto the gel. Electrophoresis was carried out at 150 V until the tracking dye front reached the end of the gel.

Detection of Proteins:

Staining with produce extract: A 60-mL solution of each specific stain was prepared by combining 10 mL from each of six separate extracts from a single species prepared independently by individual students. The combination of multiple extracts for each species was intended to minimise the risk of variation that might have resulted from their preparation at home instead of a typical laboratory environment. Following electrophoresis, gels were left intact or cut into sections, depending on the experiment. Gels or sections thereof were incubated with gentle shaking in two changes of approximately 100 mL water for five minutes each in order to remove residual SDS detergent. Gel sections were then transferred to a square plastic petri dish containing the designated stain. The dishes were covered and shaken slowly for 3 hours. Whole gels were transferred to larger containers as needed and treated in the same way. When staining gels, excess unbound stain can diffuse into the gel matrix.

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Therefore, a destaining step was carried out to wash away any unbound stain in the gel by incubation with gentle shaking in several changes of water until the water remained clear.

Staining with Coomassie Blue: The Coomassie blue stain included 0.1% Coomassie brilliant blue R-250 dye (Sigma-Aldrich), 30% methanol and 10% acetic acid and it was filtered before use. The destaining solution consisted of 30% methanol and 10% acetic acid with no dye added. Gels or sections were washed as above and then placed directly in the Coomassie blue stain overnight followed by several changes of destaining solution and then several changes of water until the water remained clear.

Sequential staining: For the quantitative experiments, gels were first stained with raspberry extract and imaged under UV light. The gels were then subjected to Coomassie blue staining as above and imaged with visible light transillumination.

Imaging:

Detection was carried out using a FluoroChem E imager (ProteinSimple) with a white light transilluminator plate for visible light imaging under the "Coomassie" setting and a UV transilluminator for the fluorescent imaging under the "Ethidium Bromide" setting. Gels stained with Coomassie blue were imaged exclusively under visible light in this study because the stain is known to be detected under visible or infrared light, with no known use under UV light (Luo et al., 2006; Smejkal, 2004)

Densitometric analysis:

Densitometry was carried out where indicated using the online version of ImageJ (Rasband, 1997-2018), which was directly accessible to the students from home at https://imagej.nih.gov/ij/index.html. Using this program, images were converted as needed to the eight-bit greyscale light-on-dark form appropriate for densitometry. Signal density measurements were then obtained for identical areas in a background section of the gel (no protein) and for the protein bands, transferred to a Microsoft Excel file and then analyzed. Means and standard deviations from triplicate bands were calculated and graphed along with individual data points using Excel.

Results

Protein Detection Using the Produce Stains:

Extracts from the five produce species were used to stain identical SDS-PAGE gel sections and these were then imaged in two ways. Fig 2A shows images obtained under visible light transillumination after staining with the five produce extracts and with Coomassie blue. The prestained molecular weight markers, which are manufactured with covalently attached dyes, were visible in lane 1 of each gel section. Coomassie blue staining, used as a positive control, also revealed many electrophoresed proteins in lanes 2-4 as expected. Both the beet and blueberry extracts stained proteins, resulting in faint, but detectable, unstained marker bands (lane 2) along with intense BSA and rat plasma protein bands (lanes 3-4). These stains also resulted in considerable background opacity in the gels. The cabbage, raspberry and strawberry extracts did not generate any protein signal using visible light and background opacity was also low with those stains. None of the stains resulted in banding or irregularities independent of protein presence, as shown by the clear lanes where there was no protein loaded (lane 5 on each gel section).

Under UV light transillumination, all five produce extracts allowed fluorescence-based detection of electrophoresed proteins (Fig 2B). The beet and blueberry extracts stained the gels more strongly

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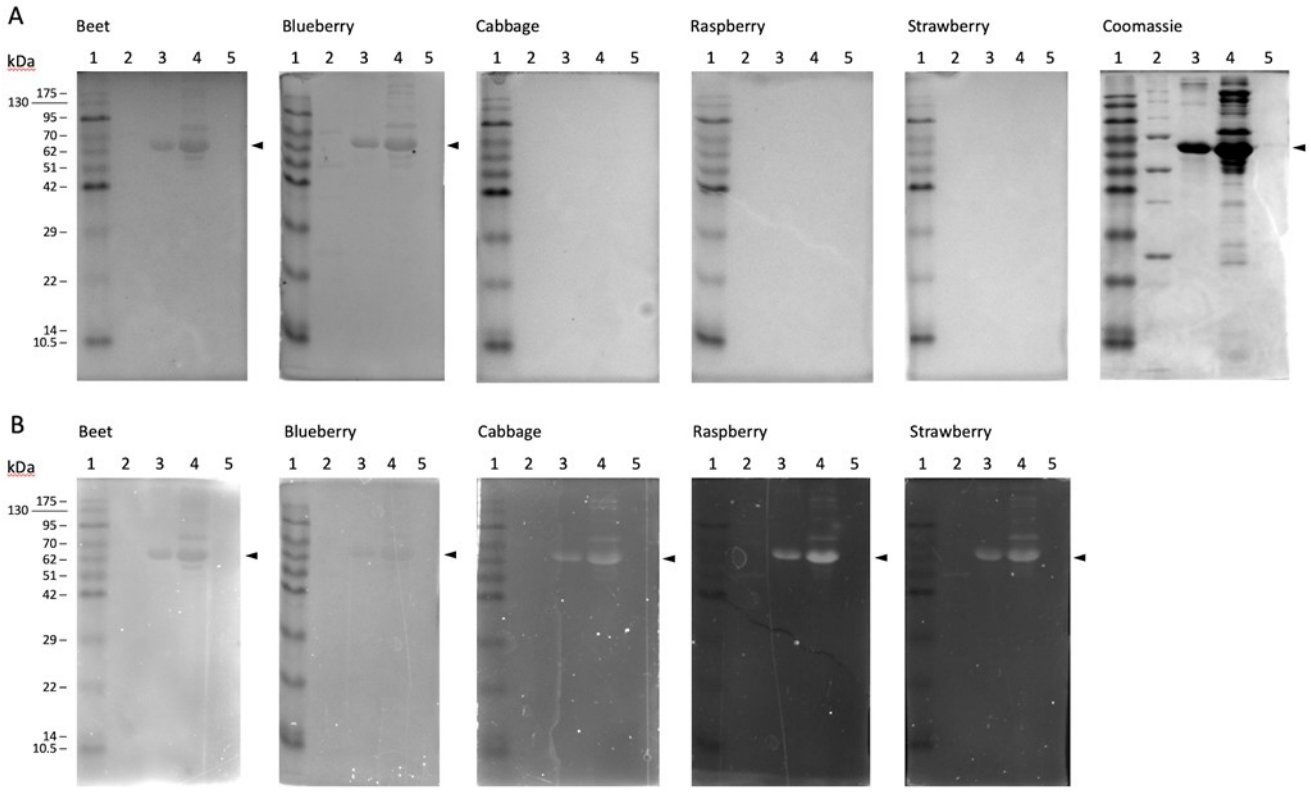

Figure 2 Staining of proteins using aqueous plant extracts. Proteins were separated by 12% SDS-PAGE and then stained using a series of aqueous plant extracts or Coomassie blue. For each gel section, lanes are as follows: (1) 5 µL pre-stained molecular weight markers, (2) 5 µL unstained molecular weight markers, (3) 5 µg bovine serum albumin, (4) 5 µg rat blood plasma, (5) no protein. Marker positions are shown on the left of the first gel section in each row. Arrowheads indicate the position of BSA when visible. Panel A: Images obtained with visible light transillumination after staining with plant extracts or Coomassie blue. Panel B: Images obtained with UV light after staining with plant extracts.

than they stained the proteins, resulting in negative protein bands in lanes 3 and 4 that were visible as a result of their lower fluorescence intensity compared to the gel background. In contrast, the cabbage, raspberry and strawberry extracts resulted in positive staining of the proteins in lanes 2-4. Strong fluorescent protein bands were observed under UV light for BSA and several other proteins using all three extracts. Furthermore, raspberry generated the lowest background fluorescence in the gel. As noted above, the protein-free lane consistently showed no signal (lane 5).

Although BSA and the corresponding serum albumin in rat blood plasma were readily identifiable, larger bands in the BSA lane suggested a slight amount of aggregated protein. The other bands in the rat blood plasma were not specifically identified except in terms of detection.

Comparison of Raspberry Staining to Coomassie Blue:

Raspberry was chosen for quantitative analysis because it resulted in a strong signal with minimal background. Triplicate samples of BSA and lysozyme were run on SDS-PAGE, stained with an aqueous raspberry extract prepared in the laboratory and imaged under UV light (Fig 3A). Subsequently, the samples were stained with Coomassie blue and then imaged using visible light (Fig 3B). Bands generated at each concentration of BSA and lysozyme were visible using both stains, although bands at the lowest concentration of lysozyme appeared faint after raspberry staining.

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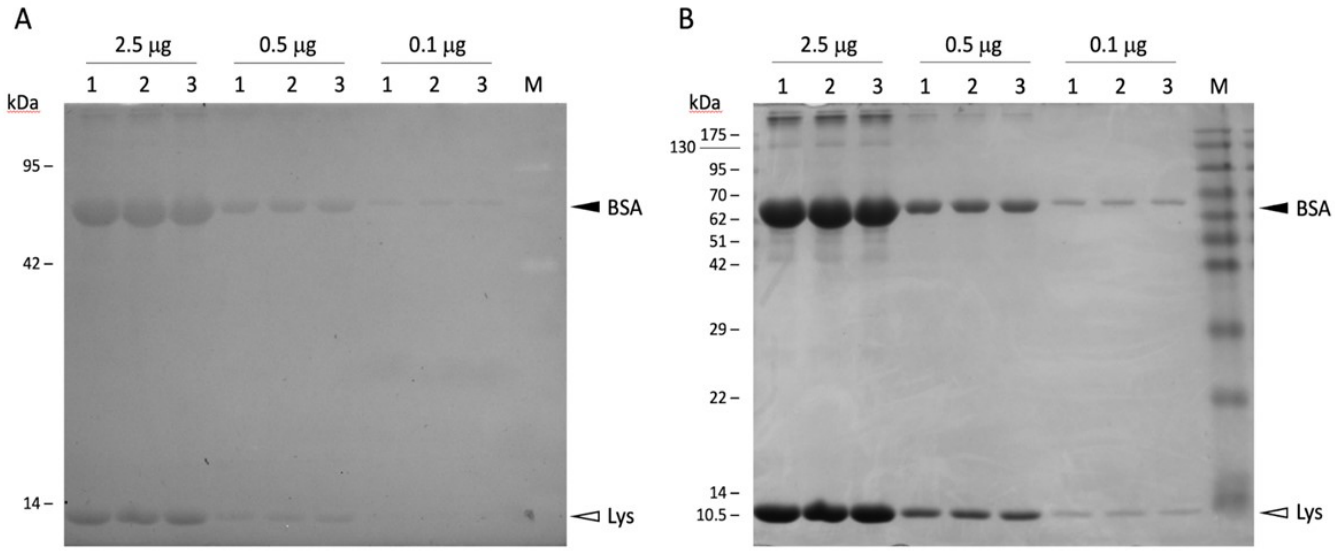

Figure 3 BSA and lysozyme separated by SDS-PAGE and then stained using raspberry extract and Coomassie blue. Each protein was loaded in triplicate in adjacent lanes of a 12% SDS-PAGE at the amounts shown. The molecular weight marker lane is denoted by M and markers positions are indicated on the left of each gel. The positions of BSA and lysozyme bands are shown by solid and open arrowheads, respectively, with labels. Panel A: Image obtained with UV light after staining with raspberry and inverted (dark-on-light). Panel B: Image obtained under visible light transillumination after staining with Coomassie blue.

Densitometric measurements performed on these bands revealed the mean signals for both BSA (Fig 4A) and lysozyme (Fig 4B) at medium (0.5 µg) and high (2.5 µg) protein masses per lane to be just under half as strong with raspberry staining as with Coomassie blue. However, the intensity was equivalent for both stains at the lowest protein level (0.1 µg per lane, Fig 4A and B). Thus, while raspberry was less sensitive than Coomassie blue when protein was abundant, it generated an equivalent densitometric signal at low protein levels, suggesting a similar limit of detection.

Discussion

Our collective responsibility for sustainability extends to the workplace, and it is a particular challenge in the life sciences laboratory (Alves et al., 2021; Sawyer, 2019), Energy expenditure, plastic consumption and toxic chemical usage are all areas to improve. A further challenge to sustainability is the regular use of chemicals that require specialized shipping (as dangerous or perishable goods) and transportation over long distances. The costs of these items and services can also be impediments to research with limited budgets. Thus, the possibility of using locally produced natural products as reagents to conduct routine research holds promise, as these might allow environmental, economic and safety advantages over more traditionally used methods. However, in order to be useful, any new product must generate reliable and interpretable data.

The results of the current study revealed visible staining of proteins by aqueous extracts of several plant species that can be grown locally in Canada. For example, staining with blueberry or beet extracts resulted in the detection of BSA and of a few other proteins in gels using visible light. Furthermore, positive fluorescent signals indicated the presence of proteins when stained with extracts from cabbage, raspberry or strawberry and detected under UV light. These findings offer practical possibilities for staining gels in any molecular

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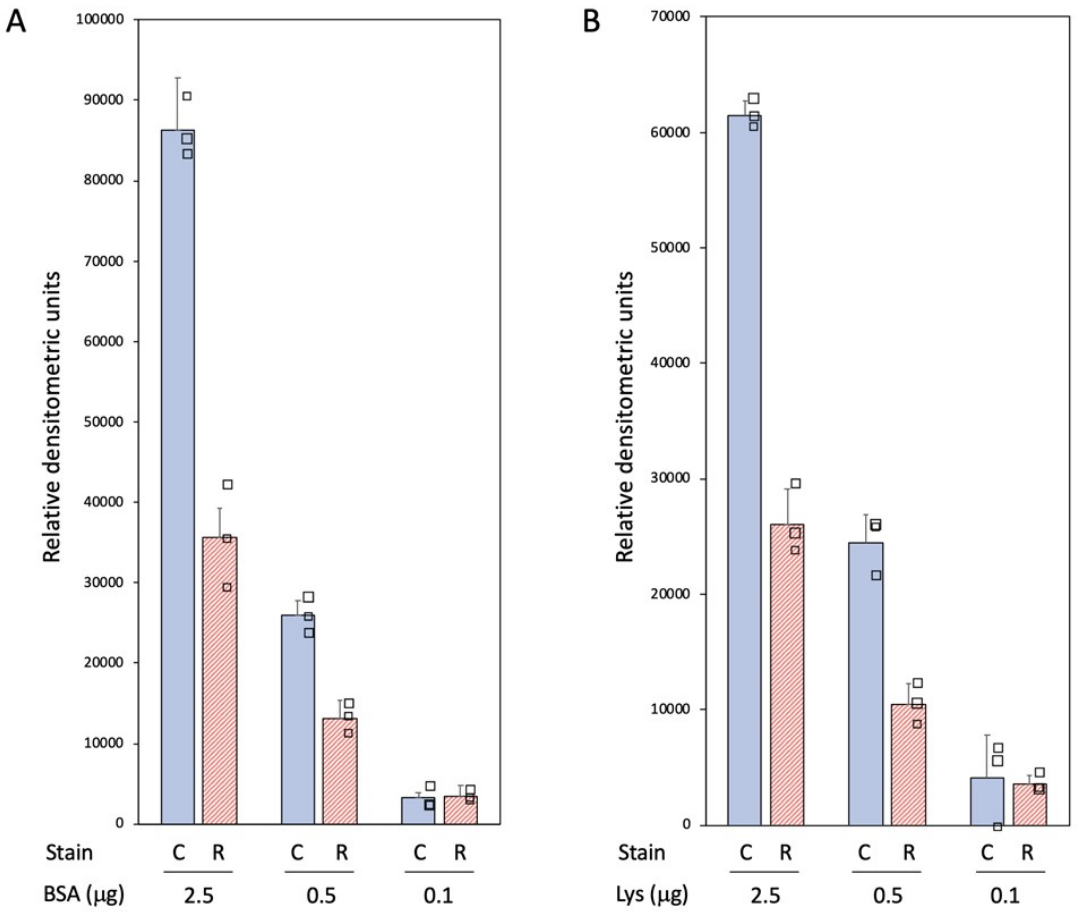

Figure 4 Densitometric signal intensities of protein bands obtained with raspberry and Coomassie blue staining on the gel shown in Figure 3. Signal intensities of protein bands after staining the SDS-PAGE gel with raspberry (hatched red bars) and Coomassie blue (solid blue bars) are shown. Bars are means ± SD with individual data points denoted as open squares. Panel A shows the results for BSA and panel B for lysozyme.

biology laboratories where UV imaging is routinely used. Nonetheless, the sensitivity and specificity of these staining extracts cannot be determined from these results. Thus, they would most likely find use in routine, repetitive laboratory procedures such as tracking the purification of a protein or screening bacterial protein expression clones. Yet, the extracts present an interesting sustainability advantage. Preparation of a staining extract from a renewable local species results in a liquid filtrate that is the stain, while the filter retains valuable products that may be useful food ingredients if prepared outside of the laboratory. In fact, a stain might be produced as a by-product of food processing. There is some risk, however, in employing extracts prepared outside of the carefully controlled manufacturing facility or laboratory environment. Thus, the use of extracts prepared as by-products or in other contexts would benefit from regular analysis by measurement of the UV/visible absorbance spectrum or of another suitable property as a means of establishing consistency among preparations. That way, any shift in staining signal could be traced to changes in preparation to avoid misleading results.

The active staining agents in the produce extracts used were not determined in this study; however, there are strong candidates in each case. Aqueous extracts of beets normally contain betalain pigments

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(Elbandy & Abdelfadeil, 2008; Udonkang et al., 2018), which have been used extensively as natural food dyes (Brudzynska et al., 2021) as well as in cosmetics and as a histology stain (Elbandy & Abdelfadeil, 2008; Udonkang et al., 2018). Thus, these molecules are likely to be the beet components staining proteins in the current work. The visible light staining of proteins by blueberry may be due to their anthocyanins, as these molecules impart the bright blue and red colours of this species (Kalt et al., 2020). Blueberry extract-stained gels had distinct blue-violet backgrounds with darker violet protein bands, consistent with a possible role for this pigment. Extracts from the other three species resulted in protein detection by fluorescence upon exposure to UV light, although the source of this effect is unknown. Water-soluble anthocyanins from red cabbage have been shown to fluoresce under UV light (Drabent et al., 1999). Conversely, in strawberries, only visible colour has been attributed to the anthocyanins, while UV-responsive fluorescence was attributed to other phenolic compounds that are colourless under visible light (Yoshioka et al., 2013). Aqueous extracts of raspberry exhibited UV-induced fluorescence emission at wavelengths ranging from 320-480 nm (Radusheva et al., 2019). Thus, there are several potential sources of the signals. The fluorescent protein-staining components are not necessarily among these. However, as several phenolics are known to interact with proteins (Buitimea-Cantúa et al., 2018), they are a reasonable candidate class.

Strong background colour was also evident in the gels incubated with the beet and blueberry stains. The components interacting with the gel appeared to be distinct from those staining the protein, as the proteins appeared as dark (clear) areas under UV light in contrast to the opaque gel. This suggests that different preparation methods or further fractionation of these extracts might separate the components causing background colour from the protein-binding stain compounds. Since commercial coffee filters vary considerably in materials, thickness and treatments (e.g. bleached or untreated), this could be an initial preparation parameter to explore in diminishing background interference while allowing full staining.

Given the intense fluorescent signal and low background of the raspberry stain in the comparative evaluation, it was chosen for quantitative evaluation. The stain rivalled Coomassie blue in the detection of bands containing very low (0.1 µg) protein, but with lower relative signal intensities at greater masses of protein. Although this weaker staining at higher protein levels may be a drawback, it could also lead to a greater dynamic range by allowing higher levels of protein to be detected without signal saturation. The two proteins used, lysozyme and BSA, have different sizes and isoelectric points and they bear no sequence similarity. Therefore, the similar staining results for these proteins using the raspberry extract is consistent with its utility as a general protein stain. The results do not rule out the possibility that particular proteins might be stained differently or to different extents with raspberry than with the widely used Coomassie or silver stains. Nonetheless, that is already a known problem when using these two standard stains. Silver staining is non-linear beyond the low nanogram range and it is prone to overstaining and saturation, resulting in an overstained pale centre in large protein bands (Patton, 2000; Weiss et al., 2009). Furthermore, neither silver nor Coomassie blue stain all proteins in proportion to their abundance (Patton, 2000; Steinberg et al., 1996).

Although the aqueous raspberry extract has effectively stained proteins in this study, there may

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be opportunities for improvement of the specificity and sensitivity of the stain. Attention to filtration, as noted above, could ensure consistency of the product and possibly reduce any background staining. Increasing the concentration of the extract, either by reducing the volume of water used or by increasing boiling or cooling/evaporation time might improve the signal. However, the incidence of background fluorescence may also vary with these changes. In addition, a centrifugation step following filtration could allow a reduction in insoluble materials and particulates. Adjusting the temperature and/or pH during extraction or staining might offer increases in sensitivity, depending on the nature of the staining molecule. Minimizing direct light exposure during preparation is a common precaution for the preservation of fluorescent molecules. Furthermore, the raspberry variety, growth and harvest conditions may affect the concentration of the staining compound present, as the fluorescence of sliced, unprocessed strawberries has been found to vary among cultivars (Yoshioka et al., 2013). Closely related local species, such as blackberries, could also be evaluated for the possibility of higher signal or lower background.

In order to maximize the utility of the raspberry stain, the possibility of frozen storage and of repeated use could be investigated. The stain from walnut husks can be reused to stain successive gels (Mushtaq et al., 2021) and it would be advantageous if this were also a possibility for raspberry-derived extracts. In addition, the compatibility of raspberry staining with subsequent mass spectrometric analysis should be evaluated, as this would also make it a versatile option for proteomic analysis and the sequencing of stained proteins.

In conclusion, aqueous extracts of five species that can be grown locally were shown to stain proteins following SDS-PAGE, with additional study of raspberry showing its potential for use in generating interpretable quantitative data. This new method is an incremental addition to sustainability in the laboratory, comparable with the goals of reducing power consumption, plastic waste and other proposed green initiatives. Moreover, across Canada, the possibility of being locally produced increases the impact of this option. Further optimization of the raspberry extract staining method and the development of new sustainable alternatives for other reagents and procedures could provide opportunities to minimize the costs and consequences of laboratory research in the life sciences while maintaining research excellence.

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