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Analytical Methods

A new colorimetric method for determination of alkylresorcinols in ground and whole-cereal grains using the diazonium salt Fast Blue RR

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ABSTRACT

A fast and inexpensive method was developed to determine the content of alkylresorcinols (ARs) in ground and whole-cereal grains. This method is based on the ability of ARs to couple with Fast Blue RR salt in alkaline medium, yielding coloured azo-derivatives that can be quantified colorimetrically. Good linearity was observed for olivetol in the range of 1–10 μg with methanol as solvent (λ_{max} = 480 nm) and 1–7 μg with butanol as solvent (λ_{max} = 530 nm). Sensitivity obtained in butanol was comparable to that obtained in the Fast Blue B based method (methanol as solvent, λ_{max} = 520 nm). In the new colorimetric method described here, incubation time was reduced to 20 min and the stability of the reaction products was as long as 3 h. The method appears promising for the analysis of 1,3-dihydroxybenzene derivatives in samples from plant breeding and food analyses.

1. Introduction

There is a strong support for the beneficial role of whole-grain intake in reducing the risk of chronic diseases, including coronary heart disease, diabetes and some cancers (Anderson, Tammy, Hanna, Xuejun Peng, & Kryscio, 2000). Phenolic compounds found in cereals bran fraction, including alkylresorcinols (ARs), were suggested to participate in such benefits on human health (Slavin, Martini, Jacobs, & Marquat, 1999). ARs are natural amphiphilic long-chain homologues of orcinol (1,3-dihydroxy-5-methylbenzene). These phenolic lipids occur in many higher plant families, bacteria and fungi (Kozubek & Tyman, 1995). They were reported in grains of cereals such as wheat, barley, rye and maize (Zarnowsky & Suzuki, 2004). Molecular structure of ARs consist of a benzene ring with two hydroxyl groups at positions 1 and 3, along with an odd-numbered alkyl chain at position 5. A wide range of biological activities have been proposed for these compounds. They are potential biomarkers in studies on the effects of whole-grain human diets on consumers' health (Ross, Kamal-Eldin, Jung, Shepherd, & Åman, 2001). In accordance with this claim, efficient and sensitive methods are needed for analyzing these compounds in whole-cereal grains, cereal products as well as in human or animal derived samples (Gajda, Kulawinek, & Kozubek, 2008).

Most general methods used for the determination of ARs are based on spectrophotometry. The results obtained from these methods are usually calculated from appropriate calibration curves prepared on the basis of weight concentrations of a standard AR analogue and are expressed in μg/g dry matter (DM). Tluscik, Kozubek, and Mejbaum-Katzenellenbogen (1981) developed a colorimetric method based on the use of the diazonium salt Fast Blue B BF_4. The method was highly specific for 5-*n*-alkyl derivatives of resorcinols with a sensitivity comprised between 1 and 10 μg of ARs. Maximum absorbance of the coloured AR–Fast Blue B products was obtained at 520 nm after 1 h incubation at room temperature. Later, Gajda et al. (2008) improved this method replacing the Fast Blue BF_4 (currently not commercially available) by Fast Blue B $ZnCl_2$ salt. This change lengthened to 3 h the stability of the products of reaction between the ARs and the diazonium salt. Sensitivity was also increased to 0.1 μg of ARs. Nevertheless, readings at 520 nm were only possible after 1 h of incubation and formation of coloured products was reduced when exposed to sunlight.

Fast Blue RR ½$ZnCl_2$ salt is often used for detection of esterase and alkaline phosphatase activities in histochemical and colorimetric analysis (Johnston & Ashford, 1980). In these reactions, naphthyl derivatives are employed as substrates and the enzymatic release of naphthol is followed via a coupling reaction with a diazonium salt such as Fast Blue RR ½$ZnCl_2$. These reactions are usually performed in basic media and the formation of coloured derivatives take only a few minutes. This situation led us to think that a colorimetric method for measuring ARs based on the use of Fast Blue RR ½$ZnCl_2$ as reagent could be developed. The aims of the present work were (1) to develop a procedure to measure alkylresorcinols based on the use of Fast Blue RR reagent (2) to determine how different factors (i.e. concentration of reagents, type of alkalinizing reagent, time of incubation) influence the reaction of Fast Blue RR with ARs (3) to determine the total

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amount of ARs in whole and ground cereal grains by the new method and comparison with the data obtained using Fast Blue B $ZnCl_2$ according to Gajda et al. (2008).

2. Materials and methods

2.1. Solvents and reagents

Methanol, ethanol, 1-propanol, butanol and acetone were from Sintorgan (Buenos Aires, Argentina). Fast Blue B $ZnCl_2$ and Fast Blue RR ½$ZnCl_2$ salts were purchased from Fluka (USA). These salts will hereafter referred to as Fast Blue B and Fast Blue RR salts, respectively. Olivetol (5-pentylresorcinol) was from Sigma–Aldrich (USA).

2.2. Cereal grains

Barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) grains were provided by Estación Experimental Agroindustrial "*Obispo Colombres*" (Tucumán, Argentina).

2.3. Extraction of ARs from cereal grain materials

Samples (80 g each) of whole and ground grains (coarsely ground in a Willey mill) of barley, wheat and maize were extracted with 80 ml of acetone for 48 h with continuous shaking at room temperature. The extracts were filtered through filter paper and evaporated under reduced pressure. Then, the dry residue was transferred with 2 ml of methanol to glass tubes. Aliquots of 200 μl (maize samples), 4 μl (wheat samples) and 20 μl (barley samples) were transferred to glass tubes, made up to 200 μl with methanol and used for the colorimetric determination (see below). All values are reported on a dry matter (DM) basis. The DM content was determined by drying the samples in an oven at 105 °C overnight, cooling at room temperature and weighing. All DM analysis were carried out in triplícate.

2.4. Colorimetric method based on the use of Fast Blue B salt

A stock solution of 0.05% Fast Blue B salt was prepared in methanol containing 1% acetic acid (Gajda et al., 2008). Fresh working solution of Fast Blue B reagent was prepared by mixing 1 part stock reagent with 5 parts methanol. Stock solutions of pure olivetol were prepared in methanol at concentration of 1 mg ml^{-1}. Aliquots of this solution comprised between 1 and 10 μl (1–10 μg, or 6.3–62.9 nmol) were placed in assay tubes and made up to 200 μl with methanol. Then, 2 ml of working solution of Fast Blue B salt was added to each assay tube. Samples extracted from cereal grains as described above, were also assayed. Absorbance was measured after 60 min at 520 nm. Each experiment was done in triplicate.

2.5. New colorimetric method based on the use of Fast Blue RR

A stock solution of 0.05% Fast Blue RR salt was prepared in methanol. Fresh working solution of Fast Blue RR reagent was prepared by mixing 1 part stock reagent with 5 parts methanol. Aliquots of the stock solution of olivetol comprised between 1 and 10 μl (1–10 μg, or 6.3–62.9 nmol) were placed in assay tubes and made up to 200 μl with methanol. Then, 2 ml of working solution of Fast Blue RR salt was added to each tube. Ten microliters of a basifying reagent was also added (see in the next paragraph below). Absorbance of the reaction mixture was measured after 15, 30, 45, 60, 90, 120, 150 and 180 min at 480 nm (Fast Blue RR salt reagent in methanol, ethanol or propanol) or 530 nm (Fast Blue B salt reagent in butanol). These wavelengths were selected because they correspond to the maximum absorbance of the coloured derivative formed in the reactions. Samples extracted from cereal grains as described above, were also assayed. Each experiment was done in triplicate.

2.6. Influence of the basifying reagent on the colorimetric reaction

Colorimetric reaction using Fast Blue RR salt was performed using different basifying reagents: 26% ammonia hydroxide, 10% sodium carbonate solution, 10% potassium carbonate solution and a mixture of 26% ammonia: 10% sodium carbonate (1:1, v/v). Each experiment was done in triplicate.

2.7. Influence of the solvent and concentration of diazonium salt on the colorimetric reaction

The influence of different solvents (methanol, ethanol, butanol and 1-propanol) was also evaluated in the reaction with Fast Blue RR salt. The same solvent was used to prepare the stock solutions of diazo reagent and their working dilutions. Each experiment was done in triplicate.

2.8. Influence of sunlight on the colorimetric reaction

To check the influence of sunlight, the incubation mixture containing Fast Blue RR salt was kept in a dark place for 60 min and its absorbance was compared with the results obtained for a mixture exposed to sunlight for 60 min. The experiment was done in triplicate, in springtime with morning sunlight.

2.9. Influence of different concentrations of diazo reagent and storage of stock solutions on the colorimetric reaction

The influence of different concentrations of Fast Blue RR on the colorimetric reaction was checked to find the optimal concentration for the highest sensitivity. In this case, 0.0125%, 0.025%, 0.05% and 0.1% (w/v) Fast Blue RR solutions were prepared in methanol, ethanol or butanol. Working dilutions were prepared using 0.05% stock reagent solutions by mixing 1 part of stock diazonium reagent with 1, 2, 3, 4, 5, 6, 20 parts of methanol, ethanol or butanol (v/v).

2.10. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and differences among means were determined through Dunnet T3 test using SPSS 7.5 for Windows.

3. Results and discussion

3.1. UV–VIS spectra of AR–diazonium coloured products obtained after reaction with Fast Blue RR and Fast Blue B salts

The spectra of the coloured products formed after reaction of olivetol with Fast Blue RR in the new method and Fast Blue B in the standard method had different shapes and wavelengths of maximum absorbance. AR–Fast Blue RR products generated in methanol, ethanol and propanol basified with K_2CO_3 had a maximum at 480 nm, while in butanol the maximum was 530 nm. Shapes of UV–VIS spectra and wavelengths of maximum absorbance of AR–Fast Blue RR products generated in ethanol or propanol were similar to those for methanol as shown in Fig. 1. Coloured products generated using Fast Blue B reagent in methanol had a maximum at 520 nm as previously reported (Tluscik et al., 1981).

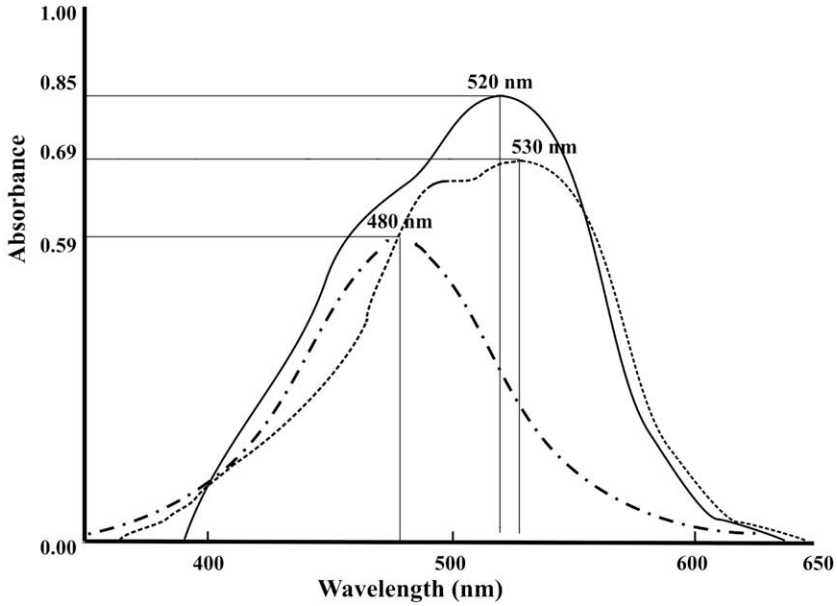

Fig. 1. UV–VIS spectra of coloured derivatives generated after reaction of 4 μg of olivetol with (a) ——— Fast Blue B salt in methanol, (b) — — Fast Blue RR salt in butanol basified with K_2CO_3 and (c) — ▪ Fast Blue RR salt in methanol basified with K_2CO_3.

3.2. Effect of basifying reagents and sunlight on the AR–Fast Blue RR products

Basifying reagents have a critical effect on coupling of diazonium salts with aryl compounds. They can affect the stability of both the diazonium reagent and the azo-dye formed by reaction of aromatic compounds with Fast Blue RR (Johnston & Ashford, 1980). For this reason, the intensity of the colour developed after addition of different basifying reagents was followed for 3 h (Fig. 2). Colour development was very close for all basifying reagents in the first 30 min. Then, the intensity decreased in all treatments, except for potassium carbonate. A white precipitate was observed in tubes basified with sodium carbonate. They could only be read after centrifugation. The response observed suggests that potassium carbonate stabilizes the formed AR–Fast Blue RR product.

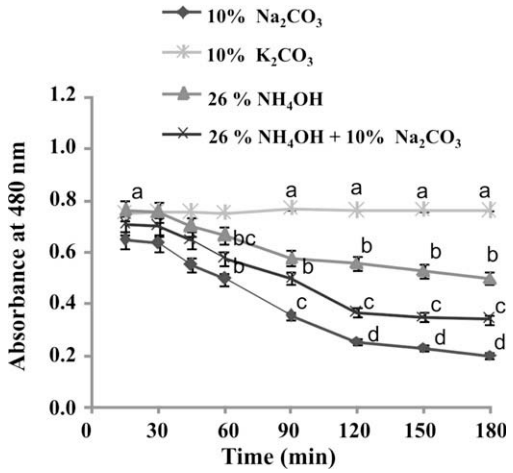

Fig. 2. Effect of basifying reagents on stability of the coloured derivative generated after reaction of olivetol with Fast Blue RR. The error bars are mean ± standard deviation ($n = 3$). Different letters indicate significant differences among means at the level of $P < 0.05$.

In a previous experiment, the green stock solution of Fast Blue RR exposed to sunlight was completely decolourated after 6 h of exposure. Nevertheless, Fast Blue RR products were formed with no difference in colour intensity between samples incubated in the dark and those incubated under sunlight. Important loses of colour intensity were observed in the method based on the use of Fast Blue B reagent when reaction was performed in presence of light (Gajda et al., 2008). Differential response observed for Fast Blue RR may be due to the short time needed for complete reaction of ARs with Fast Blue RR in basic medium. This is a valuable characteristic of the method that may allow its use in field determinations of ARs.

3.3. Influence of concentration of Fast Blue RR salt and solvent system on colour development

Butanol, 1-propanol, ethanol and methanol were assayed as solvent systems in the colorimetric reaction based on Fast Blue RR. This salt was soluble in methanol and ethanol. On the other side, Fast Blue RR was only partially soluble in both butanol and 1-propanol, and the stock solutions obtained with these last two solvents needed filtration before use. Highest intensity of AR–Fast Blue RR products was obtained at 0.1%, 0.05% and 0.025% for stock solutions prepared in methanol or ethanol, and at 0.1% and 0.05% for stock solution prepared in butanol (Fig. 3). Absorbance of coloured derivatives was also measured at different dilutions of the stock solutions of Fast Blue RR reagent. The highest absorbance was obtained by mixing 1 part of stock diazonium reagent with 4 parts of methanol, ethanol or butanol (v/v).

3.4. Influence of solvent on sensitivity and linearity of the colorimetric method

Beer's law was obeyed over the range of 0.1–6 μg olivetol (0.6–33 nmol) for Fast Blue RR in butanol and Fast Blue B in methanol (Fig. 4). Curves obtained in ethanol and methanol using Fast Blue RR reagent were less sensitive than that obtained in butanol with linearity range of 0.5–8 μg olivetol. No linear response was observed using propanol as solvent.

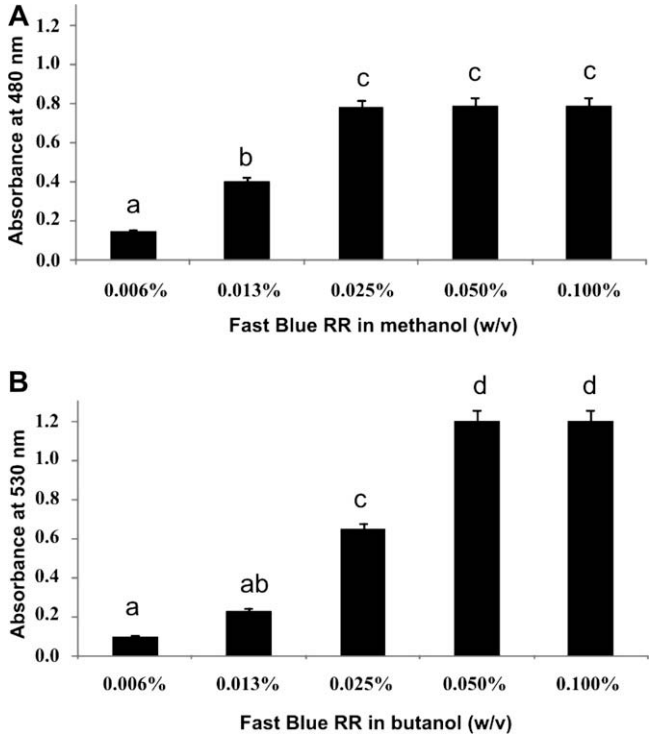

Fig. 3. Effect of concentration of stock solution of Fast Blue RR salt on the development of coloured derivatives. The error bars are mean ± standard deviation (n = 3). Different letters indicate significant differences among means at the level of $P < 0.05$.

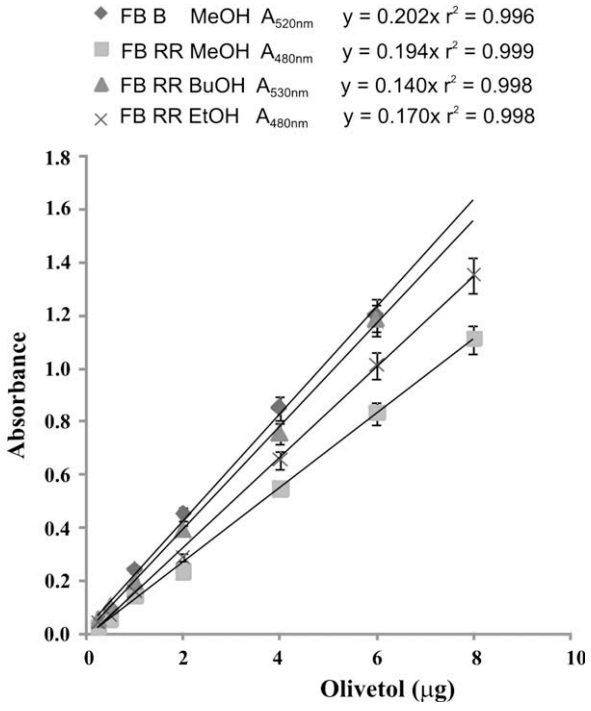

Fig. 4. Calibration curves generated for AR–Fast Blue RR products in methanol, ethanol and butanol and for AR–Fast Blue B products in methanol. The error bars are mean ± standard deviation (n = 3). FB B MeOH = Fast Blue B (methanolic solution), FB RR MeOH = Fast Blue RR (methanolic solution), FB RR BuOH = Fast Blue RR (butanolic solution), FB RR EtOH = Fast Blue RR (ethanolic solution).

Table 1
AR content in barley, wheat and maize grain material determined using Fast Blue RR reagent prepared in methanol and butanol and Fast Blue B reagent prepared in methanol.

	Total content of alkylresorcinols (μg/g of dry matter)		
	0.05% Fast Blue RR in methanol[a] (A_{480} nm)	0.05% Fast Blue RR in butanol[a] (A_{530} nm)	Fast Blue B based method[a] (A_{520} nm)
Wheat			
Whole	755 ± 11	758 ± 16	740 ± 9
Ground	732 ± 12	740 ± 11	725 ± 16
Barley			
Whole	290 ± 7	296 ± 7	285 ± 14
Ground	270 ± 11	280 ± 11	261 ± 11
Maize			
Whole	13 ± 2	10 ± 2	11 ± 1
Ground	12 ± 1	11 ± 1	10 ± 1

[a] Means of triplicates ± standard deviation, $P < 0.05$.

3.5. Content of ARs in cereal grain materials

Contents of ARs in whole and ground grains of wheat, barley and maize determined with Fast Blue RR in butanol and methanol were very similar to those obtained using the Fast Blue B based method (Table 1). The values of total ARs are similar to those previously reported (Chen, Ross, Åman, & Kamal-Eldin, 2004; Gembeh, Brown, Grimm, & Cleveland, 2001; Ross et al., 2003).

4. Conclusions

A new method was developed to measure ARs. It is based on the reaction of ARs with Fast Blue RR salt in a basic medium. Using butanol as solvent and 10% K_2CO_3 as basifying reagent, the method described here is as sensitive as the previous one based on the use of Fast Blue B reagent, having the advantage that stable AR–Fast Blue RR coloured products are obtained after only 20 min of incubation. The coloured products can be read up to 3 h later without loss of sensitivity allowing the reading of a large number of samples. The total AR content in whole and ground cereal grains determined by the new method yielded results comparable to those obtained using Fast Blue B.

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