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Short communication

Development and validation of a micromethod for fast quantification of 5-*n*-alkylresorcinols in grains and whole grain products

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ABSTRACT

A 96-well plate micromethod was developed to measure 5-*n*-alkylresorcinols (5nARs) in cereal grains and food derived products. The 5nARs reacted in alkaline alcoholic medium with Fast Blue RR ½$ZnCl_2$ salt to yield coloured azo-derivatives. The highest sensitivity for 5nARs was obtained at 490 nm with 0.025% ethanolic Fast Blue RR and 5% K_2CO_3. This reaction showed good linearity for olivetol (0.05–0.20 μg). Contents of 5nARs determined in cereal grains and derived products by the new Fast Blue RR micromethod were highly correlated (R^2 = 0.9944) with those obtained by a Fast Blue B method currently used. A Bland–Altman analysis indicated a small positive bias near to zero (R^2 = 0.0401), suggesting that the methods can be interchangeably used. The new reaction is completed in 15 min and the coloured products are read within the 15 min after completion. The micromethod offers a fast analysis of 5nARs in cereal grains and derived products with low consumption of reagents and solvents.

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1. Introduction

Epidemiological studies strongly correlate the consumption of whole cereal products with a decreased risk of chronic diseases, including coronary heart disease, diabetes and some cancers (Egeberg et al., 2009; Gil, Ortega, & Maldonado, 2011). Phenolic compounds from cereals' bran fraction, including 5-*n*-alkylresorcinols (5nARs), were suggested to participate in such benefits on human health (Slavin, Martini, Jacobs, & Marquat, 1999). The 5nARs occur in many higher plant families, bacteria and fungi (Kozubek & Tyman, 1995). They were reported in grains of cereals such as wheat, barley and rye (Zarnowsky & Suzuki, 2004). Molecular structure of 5nARs consist of a benzene ring with two hydroxyl groups at positions 1 and 3, along with an odd-numbered alkyl chain at position 5. These compounds have been suggested as potential markers for wholegrain wheat and rye in food products (Chen, Ross, Åman, & Kamal-Eldin, 2004). Analysis of 5nARs could also be used for checking contamination of nongluten containing cereals with gluten containing cereals such as wheat, rye, and barley (Ross, 2012). However, the biosynthesis of 5nARs and their resulting content in cereal grains and cereal products can be altered by many biotic and abiotic factors within different genotypes and also even within the same ecological niche (Magnucka, Suzuki, Pietr, Kozubek, & Zarnowski, 2007; Pietr et al., 2002). In accordance with these claims, fast and sensitive methods are needed for analysing these compounds in whole-cereal grains, cereal products as well as in human or animal derived samples (Gajda, Kulawinek, & Kozubek, 2008).

Several methods used for the determination of 5nARs 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 5nAR 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 5nARs. Maximum absorbance of the coloured 5nAR–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 increased 3 h the stability of the products generated after the reaction between the 5nARs and the diazonium salt. Sensitivity was also increased to 0.1 μg of 5nARs. Nevertheless, readings at 520 nm were only possible after 1 h of incubation and formation of coloured products was reduced when exposed to sunlight. Landberg, Andersson, Aman, and Kamal-Eldin (2009) optimised the use of a colorimetric method based on Fast Blue B for determination of total amounts of 5nARs appropriate for analysis of a large number of samples obtained from grain and whole grain derived products. Contents of 5nARs were highly repeatable, and the colorimetric method was properly validated with a GC-method. However, the incubation time

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required for this reaction was 4 h at 4 °C, with an additional hour at room temperature.

We recently proposed a macromethod for quantification of 5nARs on intact and ground cereal grains based on the use of Fast Blue RR ½$ZnCl_2$ salt (Sampietro, Vattuone, & Catalán, 2009). In this case, the reaction is performed in basic media and the formation of coloured derivatives take only a few minutes. Analysis of a large number of samples, however, requires the use of micromethods able to detect the 5nARs not only in grains but also in complex food matrices. The aims of the present work were (1) to develop a micromethod for fast quantification of 5nARs using Fast Blue RR (FBRR), and (2) to compare the 5nAR contents obtained by the new micromethod in cereal grains and whole-grain products with those determined by colorimetric quantification based on Fast Blue B.

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 be referred to as FBB and FBRR salts, respectively. Olivetol (5-pentylresorcinol), catechol, *trans*-ferulic acid and quercetin were from Sigma–Aldrich (USA).

2.2. Extraction of 5nARs from cereal grains, brans and flours

Grains of winter cereals were provided by the Agricultural Station of INTA Marcos Juarez (Córdoba). They included bread wheat (*Triticum aestivum* vars Bio INTA 3004, Baguette Premium and Themix-L), soft wheat (*Triticum aestivum* vars Entrada 4 and Entrada 6), durum wheat (*Triticum durum* vars Buck Platino, Buck Cristal and Buck Topacio), and rye (*Secale cereale* vars Fausto INTA, Camilo INTA, Lisandro INTA). The maize (*Zea mays*) hybrids Avant, Chaltén and Condor were provided by commercial sources. Six samples of 1 gram were separated from each winter cereal cultivar or maize hybrid. Half of these samples were ground in a Wiley mill. Intact and ground cereal samples were subsequently used for extraction of 5nARs. Cereal derived products from wheat (2 refined flours and 3 brans), rye (3 brans) and maize (3 flours) were purchased in markets from Tucumán (Argentina). Three samples (1 g each) from each one of these products were used. The samples of intact grains, ground grains, cereal brans, or cereal flours were extracted with 40 ml of ethyl acetate at room temperature under periodic hand shaking for 48 h (Ross, Kamal-Eldin, Jung, Shepherd, & Åman, 2001). Then, the organic extracts were filtered through filter paper and evaporated to dryness at 45 °C under reduced pressure. The dry residue obtained from each extract was dissolved in methanol (1 mL). All values are reported on a dry matter (DM) basis. The DM content was determined by drying three samples from each grain variety or hybrid, cereal bran cereal or cereal flour in an oven at 105 °C overnight, cooling at room temperature and weighing.

2.3. Extraction of 5nARs from whole grain pasta and baked cereal products

Commercial whole rye and wheat products were purchased from markets located in Tucumán, Argentina. They included wheat (3 bran breads, 3 bran cookies, 3 semolina cookies, 2 whole-grain noodles), rye (3 bran breads and 3 whole-grain cookies), and maize derived products (3 flours). Multigrain products (2 breads, 1 cookie type) were also included. Three samples of one gram separated from each kind of bran bread, bran cookie, semoline cookie and whole-grain noodles were ground in the Wiley mill. Each ground sample was extracted with 10 mL of 1-propanol/water (3:1, v/v) with three extractions in a boiling water bath (1×2 h, 2×1 h) (Kulawinek, Jaromin, Kozubek, & Zarnowski, 2006). Fresh solvent was used each time. The obtained extracts were combined and filtered through filter paper and evaporated to dryness at 45 °C under reduced pressure with the help of absolute ethanol to remove remaining water. The dry residue obtained from each sample was dissolved in methanol (1 mL). All values are reported on a dry matter (DM) basis. The DM content was determined by drying three samples from each kind of bran bread, bran cookie, semoline cookie or whole-grain noodles in an oven at 105 °C overnight, cooling at room temperature and weighing.

2.4. Colorimetric method based on FBB salt

Total 5nAR content in the cereals and food derived products was determined with a method based on FBB salt (Gajda et al., 2008). This reaction was selected because it provided total contents of 5nARs similar to those determined by a sensitive and accurate HPLC method (Kulawinek & Kozubek, 2008). A stock solution of 0.05% FBB salt was prepared in methanol containing 1% acetic acid. Fresh working solution of FBB reagent was prepared by mixing 1 part of the stock reagent with 5 parts of methanol. Olivetol was selected as reference compound for colorimetric quantification of 5nARs following Kulawinek and Kozubek (2008). A stock solution of pure olivetol prepared in methanol (1 mg/ml) was diluted to 20, 10, 5 and 2.5 μg/ml. Volumes of 20 μl from these solutions were placed in assay tubes and evaporated under a nitrogen stream. Then, 2 ml of working solution of FBB salt was added to each assay tube. Methanolic extracts (200 μl) from cereal grains and cereal derived products were also assayed. Absorbance was measured after 60 min at 520 nm. Each experiment was done in triplicate.

2.5. Improvement of the FBRR method

Solubility of FBRR was assayed in alcoholic solvents (methanol, ethanol, butanol and 1-propanol). Based on it, we selected methanol and ethanol for further improvement of the colorimetric reaction. Ammonia hydroxide and potassium carbonate were assayed as alkalinizing reagents. A volume of 5 μl of different concentrations of these chemicals were added to wells containing olivetol and known concentrations of FBRR salt dissolved in methanol or ethanol. The same solvent was used to prepare the stock solutions of diazo reagent and their working dilutions. Different concentrations of FBRR were also assayed in the microcolorimetric reaction to improve sensitivity. Stock solutions of 0.05% FBRR were prepared in methanol, ethanol, propanol and butanol. Then, each stock solution and dilutions of 0.025%, 0.012% and 0.006% (w/v) were assayed. Experiments for each alkalinizing reagent, solvent and concentration of FBRR were done in triplicate.

2.6. Improved microcolorimetric method based on FBRR salt

A stock solution of 0.05% FBRR salt was prepared in methanol. Fresh working solution of FBRR reagent was prepared by mixing 1 part of the stock solution with 1 part of methanol. Aliquots of 20 μl from olivetol solutions (20, 10, 5 and 2.5 μg/ml) were placed in wells of a 96-well microplates. Aliquots of 20 μl of the methanolic extracts were also placed in wells. Then, 180 μl of the working solution and 5 μl of 5% K_2CO_3 were added in each well. Controls were prepared by addition of 20 μl of methanol instead of methanolic extracts or standards. Absorbance was measured at 490 nm after 15 min. Each treatment comprised four wells and each experiment was twice repeated.

2.7. Selectivity of the diazonium salts for 5nARs

The improved microcolorimetric method was tested on equimolar amounts of olivetol, quercetin, catechol and ferulic acid, to check the selectivity of both diazonium salts for 5nARs.

2.8. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) and differences among means were determined through LSD test using SPSS 7.5 for Windows. Calibration curves for the improved FBRR and the FBB methods were obtained by linear regression analysis. Agreement between the colorimetric methods based on FBRR and FBB was assessed by linear regression analysis and a Bland–Altman plot (Bland & Altman, 1986) with XLSTAT for Excel.

3. Results and discussion

3.1. Improved microcolorimetric method based on FBRR

Conditions for the microcolorimetric reaction were improved in methanol and ethanol because FBRR salt was soluble in these alcohols and partially precipitated in propanol and butanol at all the

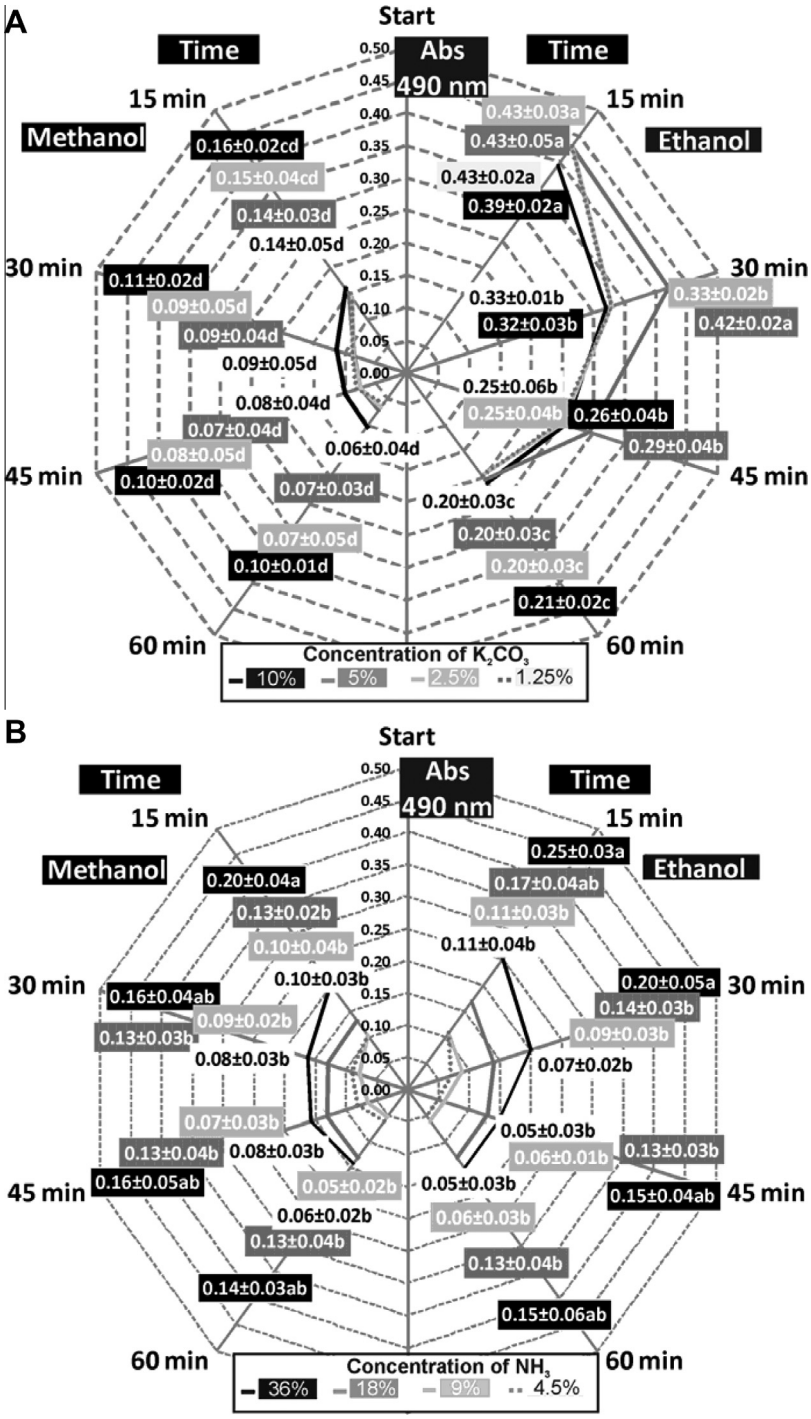

Fig. 1. Absorbance of derivatives generated by reaction of 0.4 μg of Olivetol and 0.025% FBRR in methanol (left) and ethanol (right) basified with (A) K_2CO_3 and (B) NH_3. Values are means ± SD ($n = 9$). Different letters indicate differences among means ($P = 0.05$, LSD test).

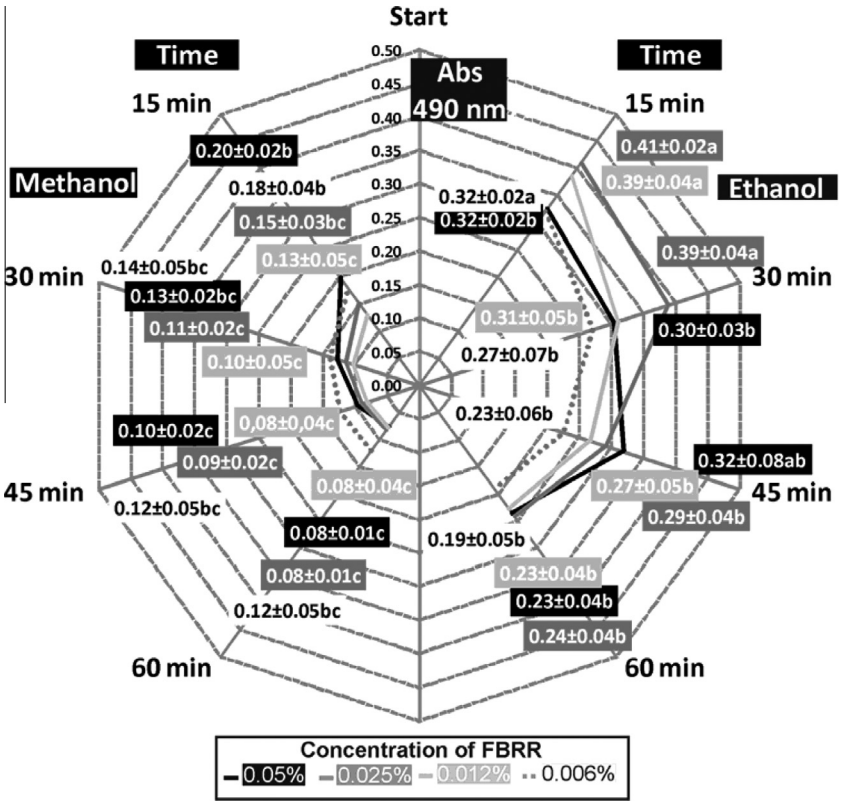

Fig. 2. Absorbance of derivatives generated by reaction of 0.4 μg of Olivetol with different concentrations of Fast Blue RR in methanol (left) and ethanol (right) basified with 5% K_2CO_3. Values are means ± SD (n = 9). Different letters indicate differences among means (P = 0.05, LSD test).

concentrations assayed. In preliminary assays, the azo-dye formed after reaction of olivetol with FBRR showed the maximum absorbance at 480 nm both in methanol and ethanol. Based on it, the micromethod was improved for 490 nm which was the nearest wavelength available in the microplate reader. Alkaline media favour the coupling reaction because the 5nARs become negative-charged nucleophilic phenoxides able to attack the electrophilic diazonium ion to form azo dyes. Addition of 5% K_2CO_3 in ethanol generated the highest readings at 15 (Abs_{490nm} = 0.43 ± 0.05) and 30 min (Abs_{490nm} = 0.42 ± 0.02) (Fig. 1A). In both incubation times the pH was 10. Lower absorbances and pHs were obtained for the same incubation times after addition of ammonium hydroxide (Fig. 1B). These lower readings were likely due to less availability of phenoxides. Readings were also low after addition of 10% K_2CO_3 (pH = 12). In these conditions, a high amount of the diazonium compound likely predominates as a diazotate ion unable to couple with FBRR (Herbst & Hunger, 2004).

The longest stability of the formed azo-dye was found in ethanolic 0.025% FBRR plus 5% K_2CO_3 from 15 (Abs_{490nm} = 0.41 ± 0.02) to 30 min (Abs_{490nm} = 0.39 ± 0.04) after starting the reaction (Fig. 2). For this reason, these conditions were selected for further quantification of 5nARs in samples of cereals and cereal derived products. The calibration curve of FBRR (r^2 = 0.99) had a smaller slope than the FBB method (r^2 = 0.98) (Fig. 3). This slope of the calibration curve of the former was lower than that of the least, This situation indicated that the last one is more sensitive than the former. Nevertheless, both colorimetric reactions obeyed to Beer's law over the same range of 0.05–0.40 μg of olivetol. Time needed for completion of the improved reaction (15 min) was remarkably shorter than that required for the FBB methods currently available (Kulawinek & Kozubek, 2008; Landberg et al., 2009). Nevertheless, coloured products generated are stable for longer time in the last ones which is an advantage for analysis of a large number of samples. This situation showed us that the use of multichannel micropipettes and a good synchronisation during preparation of the reaction mixtures is a mandatory requisite to obtain good results with the improved FBRR method.

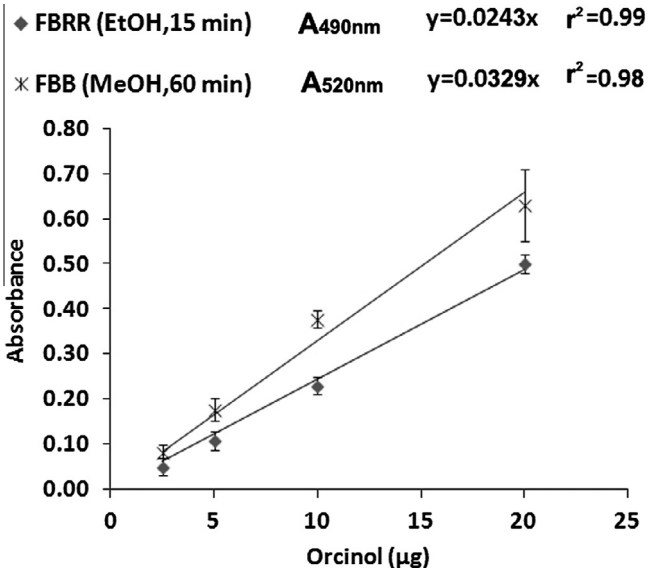

Fig. 3. Calibration curves generated for olivetol–Fast Blue RR products in methanol and ethanol (5% K_2CO_3 was the alkalinizing agent), and for olivetol–Fast Blue B products in methanol. The error bars are mean ± SD (n = 3). FBB MeOH = Fast Blue B (methanolic solution), FBRR MeOH = micromethod based on Fast Blue RR in methanol, and FBRR EtOH = micromethod based on Fast Blue RR in ethanol.

Selectivity of the colorimetric reactions was evaluated on olivetol, catechol, quercetin and ferulic acid. Fig. 4 shows absorbances

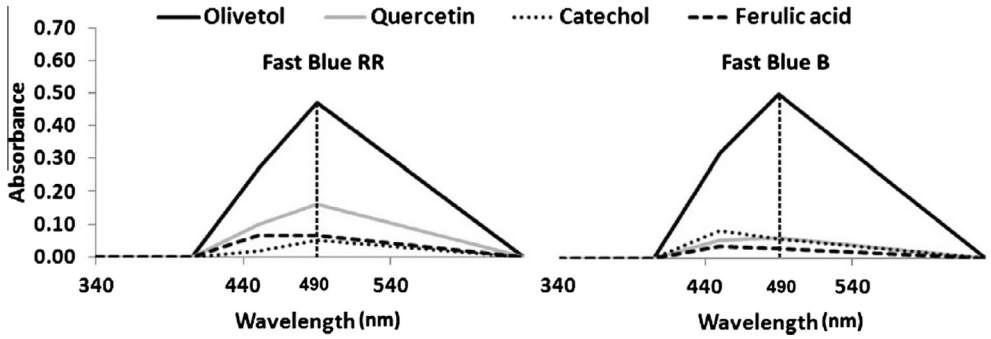

Fig. 4. Absorbance spectra of 2.2 nanomols of olivetol, quercetin, catechol or ferulic acid after reaction with Fast Blue B or Fast Blue RR.

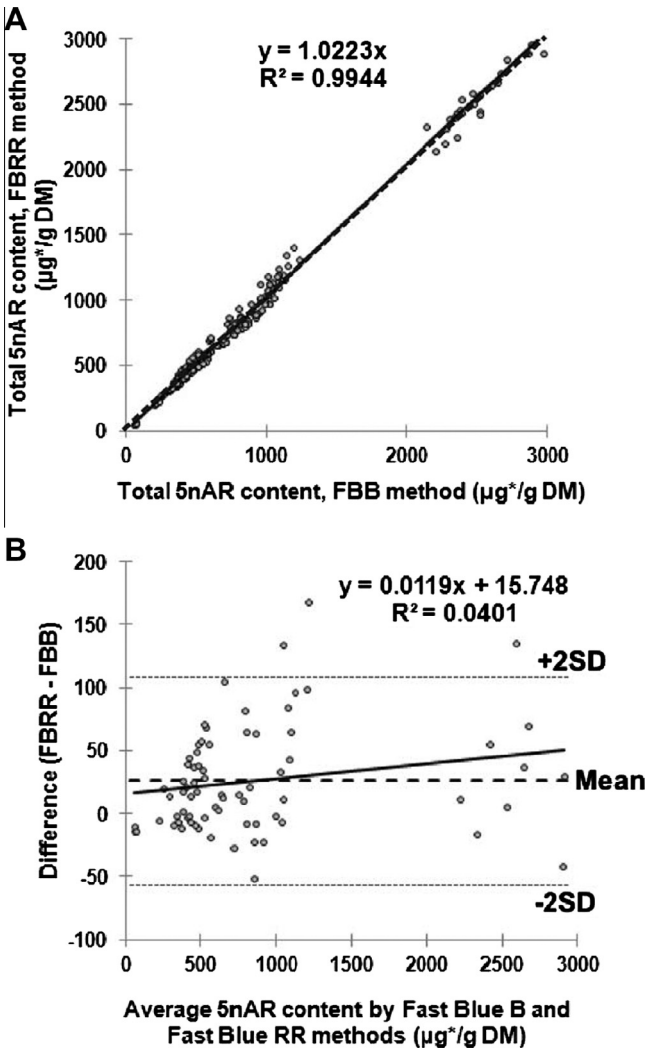

Fig. 5. (A) Total alkylresorcinol (5nAR) content in samples of cereal grains and food derived matrices determined by the improved Fast Blue RR method and the Fast Blue B method. The dotted line is the unity line. (B) The Bland–Altman plot showing the agreement between the colorimetric methods. Average of the two methods is plotted on the *x*-axis and the observed difference between the methods on the *y*-axis (FBRR–FBB), $n = 219$. (*)Total 5nAR content is expressed in µg of olivetol. DM = dry matter.

obtained by FBRR and FBB methods at equimolar amounts for these substances in the wavelengths available in the microplate reader. Both methods had no significantly different readings for ferulic acid and catechol. Quercetin reacted with the FBRR salt ($Abs_{490nm} = 0.15 \pm 0.02$) more than with the FBB salt ($Abs_{490nm} = 0.07 \pm 0.03$), indicating that the improved reaction should be cautiously evaluated on complex food matrices. Nevertheless, both methods showed the highest selectivity for olivetol ($Abs_{490nm\ FBB} = 0.51 \pm 0.02$ and $Abs_{490nm\ FBRR} = 0.46 \pm 0.03$).

3.2. Comparison of the colorimetric method based on FBB with the improved microcolorimetric method based on FBRR

Total content of 5nARs determined by the improved FBRR method was linearly regressed as independent variable against total content of 5nARs determined by the FBB method (Fig. 5A). A high agreement between both methods was observed. Slopes of the regression equations obtained with ($Y = 1.0102X + 17.321$; $R^2 = 0.9947$) and without intercept ($Y = 1.0233X$, $R^2 = 0.9944$) were slightly deviated from the unity line. The Bland–Altman plot (Fig. 5B) showed a small positive bias near to zero between the two methods ($Y = 0.0119X + 15.748$, $R^2 = 0.0401$, $P < 0.001$) which could not be associated to sample origin or processing and was of importance only for few samples with high total content of 5nARs. Taken together, these results indicate that the FBRR and FBB colorimetric methods can be interchangeably used for quantification of 5nARs.

4. Conclusions

A new microcolorimetric method was developed to quantify 5nARs in grains of wheat and rye, and their bran or whole-grain derived products. It consists in the reaction of 5nARs in an ethanolic solution of 0.025% FBRR after addition of 5 µl of 5% K_2CO_3. Total contents of 5nARs measured by the new micromethod yielded results comparable to those obtained with colorimetric methods based on the use of FBB. The new micromethod showed to be selective and sensitive enough for proper quantification of 5nARs in the grain and food grain matrices investigated. It allows the fast analysis of several samples with less solvent and reagent inputs and requires only 15 min for full completion. The coloured products can be read from 15 to 30 min after starting the colorimetric reaction.

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