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Comparison of high-performance ion chromatography technique with microbiological assay of *myo*-inositol in plant components of poultry feeds*

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ABSTRACT

An ion chromatography technique (HPAEC-PAD) was employed for determination of inositol in plant components of feeds as an alternative to a microbiological assay. The study included analysis of total, free and dialysable inositol released from samples by an *in vitro* procedure simulating digestion in the gastrointestinal tract of birds. Inositol was separated on the high-performance anion-exchange CarboPack MA1 column and monitored by the pulsed amperometric detection (PAD). As compared to the HPAEC-PAD technique, microbiological assay produced 2-3 fold lower values of total inositol, probably due to *myo*-inositol stereoselectivity of *Saccharomyces cerevisiae* ATCC 9080 analytical strain. The overall correlation coefficient was 0.989, 0.941 and 0.736 for total, free and dialysable inositol, respectively. Microbiological method of analysis rather than the HPAEC-PAD technique may be recommended for determination of dialysable *myo*-inositol released from feed samples during *in vitro* digestions that simulate gastrointestinal tract of birds. The HPAEC-PAD technique was more sensitive, convenient and suitable for total inositol determination.

KEY WORDS: poultry, soyabean meal, inositol, dialysable inositol, microbiological assay, ion chromatography

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INTRODUCTION

Plant cells metabolize *myo*-inositol *via* a number of pathways, leading to glucuronate, pectin and ascorbic acid synthesis, to the formation of sucrose and its galactinol conjugates, synthesis of phytate and *myo*-inositol phosphates that are important in signal transduction mechanisms as well as to generation of phospholipids that constitute biological membranes (Loewus and Murthy, 2000; Mueller-Roeber and Pical, 2002; Eyster, 2006; Van Meer et al., 2008). *Myo*-inositol is considered to be a growth factor for certain organisms and an indicator of abnormal metabolism or disease in humans (Holub, 1986). In rodents and fish its deficiency may be associated with fatty liver, intestinal lipodystrophy and a lack of growth. Deranieh et al. (2009) reported that the lack of *myo*-inositol in the cultivation medium inhibited proliferation and caused death of human cells *in vitro*.

Phytates - salts of phytic acid (*myo*-inositol hexakisphosphate) are the major storage form of phosphorus in cereal grains, legumes and oilseeds. In the nutrition of monogastric animals and humans these compounds are antinutrients due to the ability to chelate metal ions and to form complexes with protein and carbohydrates reducing their bioavailability (Greiner et al., 2006). Enzymatic hydrolysis of phytates by endo- or exogenous phytases leads to formation of *myo*-inositol phosphate intermediates in addition to inorganic phosphate (Viveros et al., 2000; Selle and Ravindran, 2007). D-*chiro*-inositol and its methylated form, pinitol, the major cyclitols of soyabean are also suspected to play a role in diseases prevention and health-promoting (Lee et al., 2003).

Żyła and co-workers (2004) found significantly increased body weight gains of broilers fed low phosphorus diets supplemented with 0.1 % of *myo*-inositol. A similar level of *myo*-inositol might be liberated by a synergistic action of phytase A and a fungal acid phosphatase (phytase B) on phytate molecule when poultry feeds are supplemented with both enzymes (Żyła et al., 2004). It seems possible therefore that enzymatically generated *myo*-inositol (EGI) resulting from action of phytases on feed phytates, may modulate metabolism and improve performance of growing broilers. Determination of different forms of *myo*-inositol in feeds and feedstuffs is necessary to estimate the EGI pool among different *myo*-inositol phosphates generated during enzymatic hydrolysis of phytates both *in vitro* and *in vivo*.

A couple of analytical methods have already been published for determination of *myo*-inositol in foods. These include microbiological assay (Norris and Darbre, 1956), enzymatic test (Kozuma et al., 2001), gas chromatography method (Perello et al., 2004) and HPLC procedure (Indyk and Woollard, 1994). Generally, the procedures need chemical derivatization of samples, base on indirect measurements,

are time-consuming and not completely reliable for routine analyses. Since its introduction, pulsed amperometric detection (PAD) has considerably improved the sensitivity and selectivity of separation of carbohydrates and alditiols by high-performance anion-exchange chromatography (HPAEC) (Corradini et al., 1997; Tagliaferri et al., 2000). The literature data about using the HPAEC-PAD procedure for determination of *myo*-inositol in feeds and feedstuffs are scarce, however.

From analytical standpoint, *myo*-inositol present in feeds or foods may be assayed either as a "free" inositol that is usually extracted by 0.04 M HCl or as "total" inositol determined after hydrolysis of a sample under harsh acidic conditions (1-6 M HCl, 120-140°C). From nutritional standpoint, however, it would be of interest to determine the amounts of inositol that are liberated from feeds during digestion of a sample in the intestinal tract. This might be achieved by using an *in vitro* procedure that simulates digestion in the intestinal tract of broilers and by determination of "dialysable" inositol content in feed components.

The objective of the current study was to determine the total, free and dialysable inositol contents in feed components and in fully formulated maize-soyabean meal-based and wheat-soyabean meal-based feed samples by the HPAEC-PAD technique (Technical Note, 2000) as well as by a conventional microbiological assay with *Saccharomyces cerevisiae* ATTC 9080 strain and to evaluate the methods in terms of different inositol isomers quantification.

MATERIAL AND METHODS

Material

Samples of wheat, conventional or genetically modified (GM) maize (BT maize MON-810), conventional or GM soyabean meal (RoundUpReady) and samples of fully-formulated wheat-soyabean meal-based and maize-soyabean meal-based feeds were obtained from National Research Institute of Animal Production (Balice, Poland). *myo*-Inositol, *myo*-inositol-2-monophosphate and sodium hydroxide (50%) for HPAEC were obtained from Fluka (Poland). Inositol assay medium, malt and yeast extracts, peptone, dextrose, agar were from Becton, Dickinson & Company (USA). The strain of *Saccharomyces cerevisiae* ATCC 9080 was purchased from American Type Culture Collection. All other chemicals were of analytical grade.

Enzymes

Pepsin (declared activity of 225 000 units · mg^{-1}) and pancreatin (activity

8 × U.S. Pharmakopeia) were purchased from Sigma Chemical Co (St. Louis, USA).

Total inositol determination

Proper amounts of feed or feedstuff (100-200 mg) were subjected to acid hydrolysis with 2 ml of 1M HCl in Duran® glass tubes (16 x 160 mm) for 48 h at 123ºC. After cooling the samples were prepared further according to method of Norris and Darbre (1956). Sample pH was adjusted to 4.8-5.0 and inositol content was assayed in the hydrolyzate.

Free inositol determination

Samples (1.00 g) of feeds or diet ingredients were homogenized and extracted with 0.04 M HCl according to method of Norris and Darbre (1956). Sample pH was adjusted to 4.8–5.0 and then subjected to microbiological and HPLC assays.

In vitro digestions and dialysable inositol

The *in vitro* procedure of Żyła et al. (1995) that simulates digestion in the intestinal tract of broilers was used. Briefly, the procedure comprised three consecutive incubations of samples at 40ºC and at pH 5.80 for 30 min, at pH 2.75 for 45 min, and at pH 6.10 for 240 min, that simulated digestion in the crop, gizzard and duodenum, respectively. The acidic digest contained pepsin (3000 units per gram of a sample) whereas duodenal digestion was carried out with pancreatin in dialysis bags immersed in buffered 0.1 M NaCl solution. In samples of dialysates pH was adjusted to 4.8-5.0 and inositol concentration was assessed by both methods.

Microbiological assay of myo-inositol

Maintenance of organism. The strain of *S. cerevisiae* ATCC 9080 was maintained by monthly subculturing for 24 h at 30ºC on malt-agar slants. The medium used consists of yeast extract (0.3%), malt extract (0.3%), peptone (0.5%), dextrose (1%) and agar (2%).

Basal medium. Inositol assay medium was an inositol-free dehydrated medium containing all other nutrients and vitamins essential for the cultivation of *S. cerevisiae* ATCC 9080 (Difco™ Inositol Assay Medium). The addition of inositol in specified increasing concentration gave a growth response of a test organism that was measured turbidimetrically (Norris and Darbre, 1956).

Procedure. A loopful of culture from stock culture slant of *S. cerevisiae* ATCC 9080 was removed and suspended in 10 ml of sterile NaCl solution (0.85%). Cells were centrifuged (2000 *g*, 10 min) and supernatants were decanted. Cells were washed three times with sterile 0.85% NaCl solution. One ml of cell suspension was diluted in 1000 ml of 0.85% NaCl and 40 µl portions of the suspension were used to inoculate each assay flask. The standard curve was obtained by using *myo*-inositol at concentrations from 0 to 2 µg · ml^{-1}. Following inoculation, flasks were incubated at 30ºC for 20-24 h. To stop growth of yeast flasks were placed in the refrigerator for 30 min. Growth was measured turbidimetrically at 660 nm. The concentration of *myo*-inositol in samples was calculated using only those values that did not vary more than ±10 % from the average.

Chromatographic system and detection

All chromatograms were generated using a HPLC system that consisted of a isocratic pump model ISO-3000 (Dionex, Sunnyvale, CA), a pulsed amperometric detector (Dionex Model ED50), a Rheodyne rotary injection valve with 20 µl injection loop and a column CarboPac MA1 8.5 µm particle dia. (250 x 4 mm i.d.) coupled with a guard CarboPac MAl column (5 x 4 mm i.d.). The flow-through detection cell contained a gold working electrode and a Ag/AgCl reference electrode. Acquisition and processing of chromatographic data were done by a Chromeleon PC Integration software ver. 8.0 (Dionex). All chromatographic analysis were carried out at ambient temperature with flow-rates of 0.4 ml · min^{-1} using 1 M sodium hydroxide as eluent. The sample before injection was filtered through 0.22 µm nylon syringe filters. Conditions employed here were suitable for the efficient separation and detection of sugars and alditiols (Dionex, Application Note 66). The detection mode is called integrated pulsed amperometric detection (IPAD).

Recovery experiment

Recovery of inositol standard was determined by careful, 3-step mixing of *myo*-inositol with GM soyabean meal to have final concentration of added inositol at 2 mg · g^{-1} of meal (dry substance basis) and assaying total, free and dialysable inositol concentrations by both analytical procedures.

Statistical analysis

Data were collected in triplicate and subjected to two-way analysis of variance.

In case of significant interaction between analytical method and analysed material one-way analysis of variance was employed for each method separately and differences among means were assessed using post-hoc Tuckey test of the Statistica for Windows v. 9,0 statistical package. Correlation and regression analysis was performed using appropriate modules of the same software. Statistical significance was accepted at $P<0.05$.

RESULTS

A linear relationship between detector response and *myo*-inositol concentration over 0-100 $\mu g \cdot l^{-1}$ was observed in the high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) technique. Correlation coefficient of 0.998 between *myo*-inositol concentration and signal strength of the detector was calculated with 100 ng being the lowest *myo*-inositol concentration in standard solutions. Based on a signal vs noise ratio, detection limit of 10 ng was determined for the HPAEC-PAD procedure (in the microbiological assay the limit of detection was 2 µg). The relative standard deviation of the retention time for selected standards was 0.15% for one-day analysis (data not shown). Typical chromatogram of *myo*-inositol standard (25 µg) is depicted on Figure 1 along with a chromatogram of "total" inositol extracted from a sample of maize-soyabean meal diet. On Figure 2, the chromatogram of 25 µg *myo*-inositol is contrasted with the elution pattern of 25 µg of *myo*-inositol mixed with 50 µg of *myo*-inositol 2-monophosphate. The latter

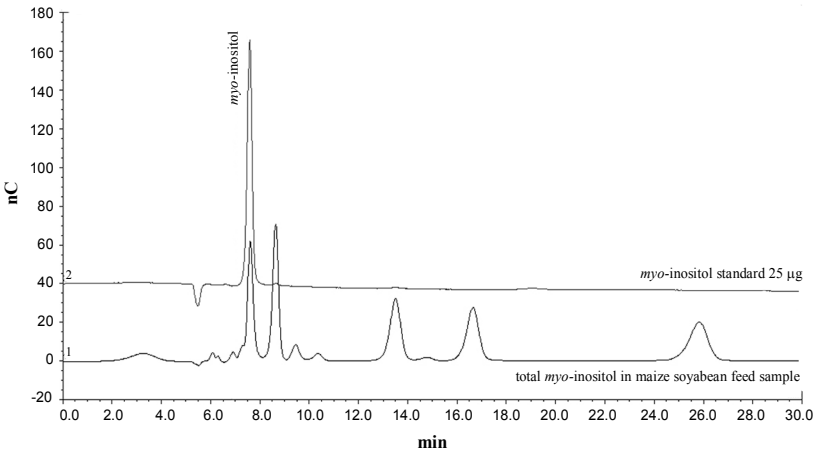

Figure 1. Overlayed HPAEC-PAD chromatograms of *myo*-inositol standard and total inositol extracted from a sample of maize-soyabean meal diet. Chromatographic conditions: column CarboPack MA-1; mobile phase 1 M sodium hydroxide. Flow rate, 0.4 ml $\cdot$ min^{-1} at room temperature, pulse amperometric mode as described in Material and methods

substance had much longer retention time and was hardly noticed by the PAD detector.

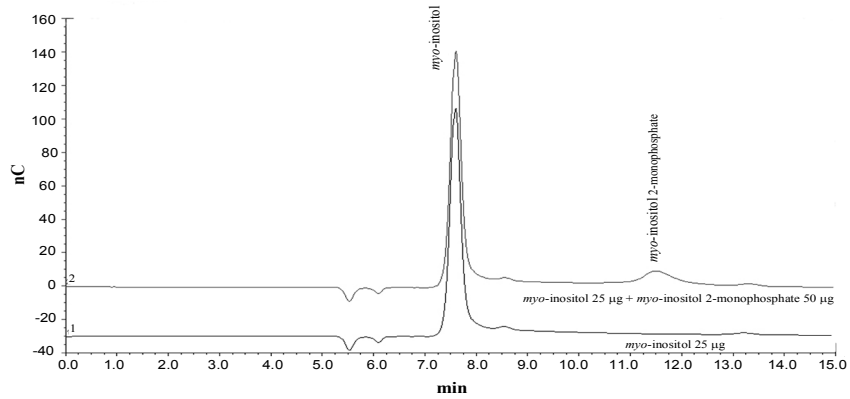

Figure 2. Overlayed chromatograms of *myo*-inositol and *myo*-inositol 2-monophosphate standards. Chromatographic conditions: column CarboPack MA-1; mobile phase 1 M sodium hydroxide. Flow rate, 0.4 ml · min^{-1} at room temperature, pulse amperometric mode as described in Material and methods

There was a significant influence of the analysed material and of the method of analysis and a significant interaction of these factors on total, free and dialysable inositol determined in feeds and feedstuffs (two-way analysis of variance). To overcome the interactions, means were calculated separately for the microbiological and HPLC methods using one-way analysis of variance (Table 1). The highest values of total inositol were determined by the HPAEC-PAD method in GM and in conventional soyabean meal samples (7957 and 5971 µg · g^{-1}, respectively). Total inositol determined in these samples by the microbiological assay amounted 2392 and 2636 µg · g^{-1}, respectively. Both analytical methods showed that conventional and GM maize samples had the lowest content of total inositol among all samples studied. Wheat-soyabean meal-based feed was found to have higher total inositol concentration than maize-soyabean meal-based feed when assayed by the HPAEC-PAD procedure but not in the microbiological assay. Both methods produced similar amounts of total inositol in GM and conventional cultivars of maize but analyses by both methods revealed a significantly higher total inositol content in GM than in conventional soyabean meal. The values of total inositol determined by the microbiological assay were systematically lower than the amounts determined by the HPLC technique and accounted for 33% in GM soyabean to 49% in wheat of the content obtained with chromatographic method.

Table 1. Inositol contents of feedstuffs and feeds analyzed by microbiological assay and HPAEC-PAD procedure

Sample	*Myo*-inositol[1], µgl ·g^{-1} DM sample											
	Microbiological assay						HPAEC-PAD					
	total	cv, %[2]	free	cv, %	dialysable %	cv,	total	cv, %	free	cv, %	dialysable	cv, %
Wheat	1196^{d} ± 128	10.7	43^{e} ± 1	2.3	146^{b} ± 13	8.9	2454^{f} ± 117	4.8	97^{e} ± 2	2.1	178^{d} ± 16	9.0
Maize	849^{d} ± 40	4.7	58de ± 7	12.1	93^{d} ± 5	5.4	2005^{e} ± 209	10.4	186^{d} ± 8	4.3	183^{d} ± 7	3.8
GM maize	961^{d} ± 13	1.4	73^{d} ± 4	5.5	98^{d} ± 3	3.1	2048^{e} ± 213	10.4	186^{d} ± 19	10.2	155^{d} ± 10	6.5
Soyabean meal	2392^{c} ± 30	1.3	189^{c} ± 15	7.9	308^{ac} ± 11	3.6	5971^{d} ± 125	2.1	3120^{c} ± 85	2.7	2216^{c} ± 304	13.7
GM soyabean meal	2636^{b} ± 59	2.2	215^{b} ± 9	4.2	251^{a} ± 25	10.0	7957^{c} ± 280	3.5	4589^{b} ± 335	7.3	3156^{b} ± 128	4.1
Wheat-soyabean meal feed	1565^{a} ± 43	2.7	128^{a} ± 12	9.4	277^{a} ± 22	7.9	3893^{a} ± 81	2.1	895^{a} ± 67	7.5	923^{a} ± 57	6.2
Maize-soyabean meal feed	1358^{a} ± 43	3.2	121^{a} ± 11	9.1	149^{b} ± 10	6.7	3438^{b} ± 138	4.0	1141^{a} ± 102	8.9	746^{a} ± 44	5.9
SEM	36.51		5.44		7.13		72.82		67.34		59.37	
P	0.0001		0.0001		0.0001		0.0001		0.0001		0.0001	
Two-way analysis of variance												
Material	0.0001		0.0001		0.0001							
Method	0.0001		0.0001		0.0001							
Material x method	0.0001		0.0001		0.0001							

[1]mean± S.D. (n=3 for microbiological assay, n=4 for HPAEC-PAD), mean values marked by the same index letter [a-f] within the column are not statistically different at level P<0.05

[2]coefficient of variation (cv)

Recovery experiment performed with a sample of GM soyabean meal revealed that 97, 91 and 85% of added standard was determined in total, free and dialysable inositol, respectively, whereas using the microbial assay 88, 98 and 99%, of total, free and dialysable inositol, respectively, was recovered.

The concentrations of free inositol when expressed as a percentage of total inositol content varied from 3.6% in wheat to 8.9% in maize-soyabean meal-based feed when assayed by the microbiological procedure whereas the HPAEC-PAD method produced a variation ranging from 3.95% for wheat to 58% for GM soyabean meal. Generally, in samples containing soyabean meal, be it conventional or GM, the ratio of free to total inositol was much higher when determined by the chromatographic procedure. The concentrations of dialysable inositol determined by the microbiological method were significantly higher than those found for free inositol and accounted for 10.9 to 17.7% of total inositol. Furthermore, the difference between these two forms was higher in samples containing wheat than in samples comprising maize. Surprisingly, these observations were not confirmed by the HPAEC-PAD procedure where dialysable inositol concentrations determined in majority of samples were significantly lower than corresponding free inositol levels. In contrast to microbiological assay, the chromatographic determination of free an dialysable inositol resulted in enormous variation of their contents ranging from 97 to 4589 $\mu g \cdot g^{-1}$ (free inositol) and 178 to 3156 $\mu g \cdot g^{-1}$ (dialysable inositol) for wheat and GM soyabean meal, respectively. Comparable amounts of free inositol were assessed by both methods in fully-formulated wheat-based diet and maize-based diet, but microbiological assay gave significantly higher dialysable inositol in wheat-based feed. There were no differences in free inositol or in dialysable inositol between samples of conventional and GM maize.

Regression analysis of the data generated by the two analytical procedures revealed a highly significant relationship ($P<0.05$) for the combined data of total, free and dialysable inositol with the correlation coefficient of 0.843 (data not shown). The relationship between these two data sets was much stronger when samples of conventional and GM soyabean meal were excluded from the analysis ($r=0.962$; Figure 3). For the particular forms of inositol that were analysed in this work correlation coefficients of 0.98, 0.94, and 0.74 for the total, free and dialysable inositol, respectively, were calculated whereas after excluding soyabean samples the values found were 0.92, 0.93, and 0.82, respectively.

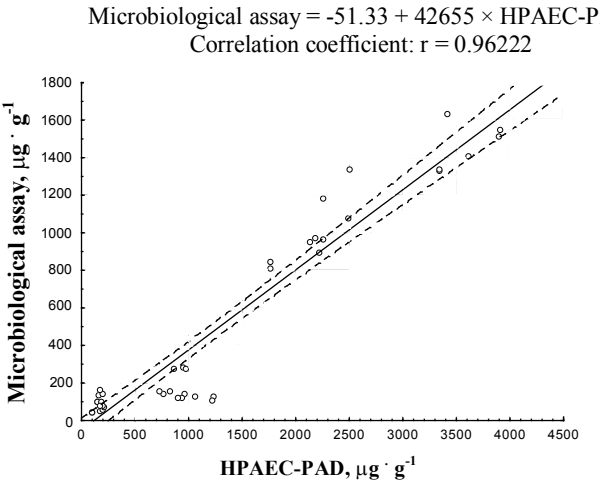

Figure 3. Correlation between inositol concentrations determined by microbiological assay and HPAEC-PAD technique after excluding samples of soyabean

DISCUSSION

Microbiological and liquid chromatography methods of total inositol determination have already been compared during analysis of dietetic milk powders (Tagliaferrii et al., 2000). Similarly to our findings, the authors reported systematically higher concentrations of total inositol determined by the HPAEC-PAD technique (420 to 1340 μg · g^{-1}) than by the microbiological procedure (270 to 1120 μg · g^{-1}). Our findings clearly indicate, however, that this discrepancy cannot be attributed to different extraction methods or to inability of *S. cerevisiae* ATCC 9080 strain to assimilate inositol monophosphate, as was thought before. In the study presented here, the same acidic extract was applied, after neutralization, to both analytical methods. Furthermore, even in the mild extraction conditions applied for free inositol determination, the difference between values given by the two methods was striking. To the contrary, Indyk and Woollard (1994) reported similar amounts of free inositol in skimmed milk powders, whole-milk powders and milk infant formulas, determined by a HPLC and a microbial procedure with *Saccharomyces uvarum*. In our HPAEC-PAD method, *myo*-inositol monophosphate did not co-elute with *myo*-inositol as shown on Figure 2. Considering the very similar stereochemistry of *myo*- and *chiro*-inositol, both isomers may be expected to have the same retention time. It seems therefore that the only idea that can be offered for the explanation of differences in total inositol as well as in free inositol given by the two methods tested here is co-elution of inositol isomers other than *myo*-inositol from the Carbopack MA1

column, on the one hand, and *myo*-inositol stereo-selectivity of the analytical *Saccharomyces* strain used in microbiological procedure, on the other. Kong et al. (2008), with the help of capillary electrophoresis coupled with electrochemical detection, successfully separated *chiro*- from *myo*-inositol in samples of black rice bran and reported that the D-*chiro*-inositol may contribute 30 to 44% of both isoforms in different varieties of rice. Soyabean seeds are known to contain up to 0.9 % of pinitol, a methylated form of D-*chiro*-inositol that can be separated from D-*chiro*- and *myo*-inositol by gas chromatography (Lee et al., 2003; Gomes et al., 2005). In our study, high proportion of free to total inositol in samples of soyabean meal determined by the chromatographic procedure was not confirmed in the microbiological assay. This finding suggests again stereo-selectivity of the *Saccharomyces cerevisiae* ATTC 9080 strain. Pinitol and D-*chiro*-inositol, the predominant cyclitols of soyabean seed coat, most probably co-elueted from the analytical column with *myo*-inositol while microbiological assay determined *myo*-inositol only.

Soyabean meal is known to be high in protein and soyabean meal samples that underwent *in vitro* digestions simulating the intestinal tract of birds were high in sugars in addition to amino acids (Żyła et al., 1995). Surprisingly, with a few exceptions (wheat, wheat-soyabean diet), the concentrations of dialysable inositol determined by the chromatographic method were lower than the amounts of free inositol. The microbiological assay, on the other hand, produced values of dialysable inositol, at least a few percentage points higher than free inositol. The differences between dialysable and free inositol where the highest in wheat and wheat-based diet samples that are known for high endogenous phytate-degrading enzymes activities whose action may release *myo*-inositol from phytate during digestion (Żyła et al., 2004). Eggleston (1999) reported that hydrophobic amino acids act as foulants of the gold electrode used in pulsed amperometric detection and lead to overestimation or to suppressive quantification of different sugars and cyclitols, including inositol. The author recommended changing of column and reducing ionic strength of eluent as possible solutions. Unfortunately, in our work, none of these remedies produced significant improvements. Regressions analysis of data obtained in our study clearly indicate that soyabean meal samples, and particularly those that underwent *in vitro* digestions, significantly lowered correlation between the two methods tested. It should be emphasized therefore that the HPAEC-PAD chromatographic procedure cannot be recommended for quantification of enzymaticaly generated inositol, i.e. *myo*-inositol released from diet components by the action of phytate-degrading enzymes. Norris and Dabre (1953), using a strain of *Schizosaccharomyces pombe* for microbiological determination of inositol in plant tissues reported total inositol concentration in soyabean meal at 5980 $\mu g \cdot g^{-1}$. Interestingly, we found quite similar level of this

compound (5971 μg · g^{-1}) using the HPAEC–PAD method, but more than 2-fold lower concentration (2392 μg · g^{-1}) was determined by microbiological assay. It may be speculated again, that in contrast to *Saccharomyces cerevisiae* ATCC 9080, *Schizosaccharomyces pombe*, originally employed as a testing strain in inositol assays was not stereoselective.

Amounts of total inositol determined by Koning (1994) in wheat-based products by gas chromatography method ranged from 2400 to 9500 μg · g^{-1} for wheat flour and wheat bran, respectively, whereas free inositol was found to amount 80 to 290 μg · g^{-1}, respectively. The values found for wheat flour are comparable to data generated in our studies by the HPAEC-PAD method that comprised all cyclitols rather than *myo*-inositol exclusively. The amounts of inositol that may be determined in wheat-based products range from 40 μg · g^{-1} (free inositol in this study, microbiological assay) to 11500 μg · g^{-1} in stone-ground wheat bread (total inositol; Clements and Darnell, 1980). In our study, dialysable inositol content in wheat was 146 μg · g^{-1} when assayed by microbiological method and 178 μg · g^{-1} with the HPAEC-PAD procedure. This indicates inositol bioavailability from wheat to be 7 to 12%. Comparable values characterized conventional and GM maize. There seems to be a possibility of substantial increase in bioavailability of inositol from feed components when exogenous phytate-degrading enzymes of microbial origin are added to diets. Diet components with high phytate content may be rich sources of enzymatically generated *myo*-inositol whereas soyabean meal seems to be a good supply of highly available cyclitols other than *myo*-inositol.

CONCLUSIONS

In conclusion it may be postulated that *myo*-inositol released from plant diet constituents that are high in phytate can be followed by microbiological assay rather than by ion chromatography technique coupled with electrochemical detection. In determination of inositol liberated from soyabean-containing samples particularly those that were subjected to multiple *in vitro* digestions electrochemical detection may not be employed and microbiological assay with *Saccharomyces cerevisiae* ATCC 9080 will not account for changes in the concentrations of *chiro*-inositol or pinitol. The chromatographic method, on the other hand, was much more sensitive and seems to be more convenient and suitable for routine total inositol determination.

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