Interference of Amino Acids in Pulsed Amperometric Detection of Peanut Sugars[1]

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ABSTRACT

High performance anion exchange chromatography and pulsed amperometry were used to separate and quantify peanut sugars extracted with methanol: chloroform:water (60:25:15, V/V/V), a highly polar solvent which solubilizes other seed components including amino acids. Free sugars were separated on an anion exchange column using a sodium hydroxide gradient and detected with a pulsed amperometric detector equipped with a gold electrode. Free amino acids in the seed extract interfered with sugar analysis by causing peak shifting and co-elution of some amino acids with sugars. Free arginine co-eluted with inositol resulting in a peak area which was 42% of the actual total area for both compounds. Proline co-eluted with fructose. Serine eluted on the leading edge of sucrose. Peak areas of these interfering amino acids were either "additive" or "subtractive" to sugar peaks. Altering the gradient elution or using cation syringe filters to remove contaminant amino acids permitted the accurate identification and quantification of peanut seed sugars. Results from this study suggest applications that can be applied in other biological systems containing free amino acids and sugars.

Key Words: Sugars, amino acids, amperometric detection, peanuts.

Roasting, deep fat frying, and other types of thermal food processing, produce products with unique flavors and aromas derived from precursors present in the raw product (Chiou *et al.*, 1993; Grosch, 1993; Leino *et al.*, 1993; Vasundara and Parihar, 1980). The nutty aroma perceived when peanuts are roasted, for example, has been attributed to the production of nitrogenous organic compounds called pyrazines (Manson *et al.*, 1966; Johnson *et al.*, 1971). Precursors of these compounds in peanuts are endogenous free sugars and free amino acids (Newell *et al.*, 1967) which serve as sources of carbon and nitrogen for pyrazine formation. Although other seed components such as lipids, phospholipids, and carbonyls (formed as a result of the Strecker degradation of amino acids) potentially contribute to the overall flavor of roasted peanuts (Johnson *et al.*, 1971), the roasted flavor potential of any lot of raw peanuts may depend largely on the free amino acid and free sugar content in the seed. The concentrations of free sugars and free amino acids in peanut seeds are influenced by factors such as plant variety, environmental conditions during plant growth, seed maturity, postharvest handling, and processing. Therefore, detection and quantification of these precursors are important when evaluating roasting potential of the raw product.

Historically, peanut sugars have been derivatized using silyl derivatives, separated on nonpolar GLC columns and detected by FID (Oupadissakoon *et al.*, 1980; Pattee *et al.*, 1981). However, significant qualitative and quantitative differences in peanut seed sugars have been reported in literature. Basha (1992) reported glucosamine, sucrose, raffinose, and stachyose in unprocessed raw seed. Other researchers have reported the presence of inositol fructose, glucose, sucrose, raffinose, stachyose, verbascose, and ajugose (Tharanathan *et al.*, 1975; Oupadissakoon *et al.*, 1980). Based on the fat-free meal, Holley and Hammons (1968) found sucrose content to vary from 2 to 6% depending on the cultivar, whereas Newell *et al.* (1967) found approximately 9%. Similar variations were reported for glucose and fructose.

Most literature on peanut sugars has dealt with qualitative and quantitative differences in mature peanut seed; however, other researchers have studied the effects of maturation and postharvest curing on carbohydrate content in seed. Ross and Mixon (1989) used a GC method to determine the free sugars in peanuts and reported that all the sugars decreased in concentration with maturation. Vercellotti *et al.* (1994, 1995) examined peanut sugars with PAD detection and confirmed relative qualitative and quantitative data with GC-MS. They recognized the potential for interference of compounds

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such as amino acids and peptides. The work reported here identifies some of the specific potentials for interference in these analyses.

Some of the differences in reported values of peanut sugars probably can be explained on the basis of differences in peanut cultivars, maturity, location, curing practices, and soil temperature effects (McMeans *et al.*, 1990); however, isolation, separation and detection methods for peanut sugars may have contributed also to these variations. Therefore, an alternative method should be used for sugar determinations which does not require derivatization of the sugar molecule.

Rocklin and Pohl (1983) described a procedure for the analysis of free sugars which consisted of separation by high-performance anion exchange chromatography (HPAC) followed by pulsed amperometric detection. Since carbohydrates have pK values from 12 to 14, retention is possible on a strong basic hydroxide form anion exchange column. Pulsed amperometric detection (PAD) is a highly sensitive detection method for carbohydrates (lower limit <10 pmol) when used at the recommended applied potential settings. We applied this method to the analysis of free sugars commonly found in peanut seed extracts. Sugars and amino acids are highly polar molecules and require a highly polar solvent for extraction. These two classes of compounds cannot be separated effectively by liquid-liquid extraction. In any analytical procedure for the analysis of sugars, the potential exists for the interference from other polar compounds in the extract. In our laboratory we found that with the HPAC-PAD method, amino acids interfered with carbohydrate separation and quantification in peanut extracts by co-eluting with specific free sugars. No information was found in the literature relating to the interference of free amino acids in the PAD analysis of free sugars.

This paper reports the separation and detection of peanut sugars using high-performance anion exchange chromatography and pulse amperometry, with special emphasis on minimizing the effects of amino acids in peanut extract by (a) altering gradient elution conditions to avoid peak co-elution, or (b) removing contaminant amino acids from the peanut extract by filtration with cation syringe filters.

Materials and Methods

Individual sugar standards were obtained from Aldrich Chemical Co., Milwaukee, WI. Chloroform, methanol, hydrochloric acid, sodium hydroxide, and HPLC-grade water were obtained from Fisher Scientific, Atlanta, GA. Cation syringe filters (25 mm) were purchased from Bio-Rad Laboratories, Hercules, CA. Amino acids were obtained from Pierce Chemical Co., Rockford, IL. Peanut seeds used in this study were germplasm samples obtained from peanut breeders and/or virginia-type peanuts (cv. NC 7) grown at the Peanut Research Station, Lewiston, NC. The experimental process was replicated three times.

Extraction of Free Sugars. About 35 g of whole peanut seed were ground into a meal. A 2-g sample of the meal was placed into a 50-mL glass tube and 30 mL of hexane/diethylether (60:40, V/V) was added to the tube. The tube was tightly capped, vortexed, and shaken on a platform shaker for 30 min, removed, and centrifuged at 1600 rpm for 15 min. The supernatant was discarded and the meal was extracted two more times with hexane/diethylether. The resulting pellet was extracted with 25 mL of chloroform/methanol/water (60/25/15, V:V:V) and blended for 1 min at high speed in a Tekmar Tissumizer™ (Tekmar Co., Cincinnati, OH). The supernatant was evaporated to dryness, the residue was resuspended in deionized water, and centrifuged at 14,000 rpm for 10 min. The supernatant was used for high performance anion exchange chromatography-PAD analysis of free sugars. Samples not immediately analyzed were stored as a dried residue at -20 C.

Chromatographic Analysis. Peanut sugars were separated using a Dionex 4000i ion chromatograph equipped with a CarboPac1™ (Dionex Corporation) anion exchange column (4 x 250 mm), CarboPac1™ guard column (4 x 50 mm), gradient pump, 20 mL injection loop, and pulsed amperometric detector with flow-through detector, gold working electrode, and Ag/AgCl reference electrode (Dionex Corp., Sunnyvale, CA). Voltage was delivered to the electrochemical cell by a potentiostat in a series of three applied potentials: E1 = 0.05 V, E2 = 0.6 V, E3 = -0.6 V with three pulse durations: t1 = 480 ms, t2 = 120 ms, t3 = 60 ms, respectively, in a repeating sequence as recommended for carbohydrate detection by the manufacturer. Eluents were degassed continuously with industrial grade compressed helium. A dilute NaOH solution was used to separate standard sugar solutions and peanut sugars (Table 1). The anion exchange column was equilibrated with A binary solvent system consisting of 90% H_2O and 10% of a 200 mM NaOH solution. Complete elution and separation of peanut

Table 1. Gradient program for peanut sugar analysis.

Time	H_2O	NaOH[a]	Curve[b]
min.	%	%	
0.0	90	10	5
7.0	90	10	5
15.0	0	100	5
23.0	0	100	5
25.0	90	10	5
31.0	90	10	5

[a]200 mM NaOH.

[b]Gradient curve Dionex software.

Table 2. Gradient program used to separate co-eluting amino acids and sugar peaks.

Time	H_2O	NaOH[a]	Curve[b]
min.	%	%	
0.0	90	10	5
2.0	90	10	5
20.0	20	80	5
20.1	0	100	5
25.0	0	100	5
26.0	90	10	5
31.0	90	10	5

[a]200 mM NaOH.

[b]Gradient curve Dionex software.

sugars was achieved by operating in the isocratic mode for 7 min and ramping the gradient from 90% H_2O to 100% 200 mM NaOH for the next 8 min. This concentration of NaOH was maintained for the next 10 min to elute oligosaccharides present in the sample. Solvent composition was then changed to 90% H_2O and 10% NaOH to re-equilibrate the column for the next injection. The gradient shown in Table 2 was used to separate co-eluting compounds by starting the gradient a 2 min and ramping from a 90% H_2O and 10% 200 mM NaOH to a 20% H_2O and 80% 200 mM NaOH solution at 20 min. However, removal of the interfering amino acids was found to be a better alternative solution to the problem. The flow rate was maintained at 1 mL/min at all times during the analysis. Aqueous stock solutions (1 mM) of sugar standards were prepared and stored at -20 C. The chromatographic system was calibrated and method files developed using an external sugar standard (diluted from stock) containing 0.1 nM each of inositol, glucose, fructose, sucrose, raffinose, and stachyose. The injection volume of all standard solutions and peanut extracts was 20 mL (contained 0.08 mg of dried material). Peak areas were quantified using a Spectra Physics Model SP 4270 integrator (Spectra Physics, Piscataway, NJ) or Dionex AI-450 integration software.

Detection and Removal of Interfering Substances. Interfering amino containing compounds in the peanut sugar extract were detected and removed by the following procedure. A sample of the extract was injected onto the anion exchange column and the effluent was directed from the column through a heated coil (130 C) with ninhydrin and the absorbance measured at 520 nm. The resulting chromatogram revealed the presence of amino containing compounds in various proportions. Cation syringe filters (25 mm) were used to remove amino acids from the peanut seed extract. The filters were prepared for use by washing with 10 mL of methanol, followed by a 10-mL rinse with deionized water and a final wash with 0.01 M HCL (approximately 10 mL) until the effluent reached pH 2. The dried sample extracts were diluted to 50 mL (contained 200 mg of dried material and 0.08 mg/20 mL), and a portion of this diluted sample was forced through the conditioned cation syringe filter. The filtrate from the syringe was injected onto the anion exchange column and the effluent directed through the ninhydrin detection system. Amino compounds were absent from the resulting chromatogram. Twenty amino acid standards were injected individually onto the anion exchange column using the chromatographic conditions for peanut sugar separation. Triplicate injections were made with all of the 20 amino acids, and their retention times and responses to the PAD detector were recorded.

Results and Discussion

Table 3 lists the retention times and area response for amino acids, and retention times and area response for free sugars on the CarboPac1™ column. Five of the amino acids gave no response with the PAD detector (Table 3). Glycine did not give a response with the PAD detector, possibly because ionization was not sufficient due to its low pK value. Aspartic acid, glutamic acid, tyrosine, and tryptophan eluted on an anion exchange column after histidine. Because the gradient used for the separation of free peanut sugars was a weak NaOH solution, a stronger NaOH solution may have been required to elute these amino acids. Based on area response and retention times, the amino acids arginine, proline, and serine were identified as amino acids which could potentially interfere with identification and quantitation of peanut sugars. Arginine co-eluted with inositol, proline eluted with fructose, and serine eluted on the leading edge of sucrose.

Table 3. Amino acid and sugar retention time on a carbopac1 column and response of amino acids to a pad detector.[a]

Amino acid	RT	Response x 10^5	Sugar[b]	RT	Response x 10^5
	min.	area counts		min.	area counts
ARG	1.68	85.03718	Inositol	1.77	65.96304
LYS	7.48	198.26671	Galactose	11.93	54.52575
GLN	12.38	143.95979	Glucose	12.58	51.88446
VAL	13.17	28.88776	Fructose	14.62	41.60484
ASN	13.40	Off scale	Sucrose	17.13	43.60258
ALA	13.70	1.43634	Raffinose	22.35	74.25552
ILE	14.02	35.83190	Stachyose	23.87	102.80258
LEU	14.35	43.04101			
THR	14.50	Off scale			
CYS	14.60	Off scale			
PRO	14.83	71.79010			
MET	16.03	77.95010			
SER	16.80	Off scale			
PHE	20.18	146.57669			
HIS	26.68	646.17904			
ASP		NR[c]			
GLU		NR			
GLY		NR			
TYP		NR			
TYR		NR			

[a]Twenty µL of a 2.5-mM solution of each amino acid injected onto column.

[b]Ten µL of a 200-mM solution of each sugar injected onto column.

[c]No response.

Many classes of electroactive compounds including carbohydrates, alcohols, aldehydes, and amine containing species such as amino acids can be detected using pulsed amperometric detection. The selectivity of detection by pulsed amperometry is based on differences in the redox potential between classes of compounds. This selectivity is diminished, sometimes substantially, in solutions containing two or more species that give similar electroconductivity responses upon contact with the gold electrode. Therefore, appropriate cleanup procedures must be used on seed extracts suspected of having electroactive species other than sugars when using a PAD detector.

Effects of Amino Acids on Sugar Quantification. Free amino acids had either an "additive" or "subtractive" effect on the quantification of specific sugars in peanut meal extract. Arginine was added to a standard sugar solution (Fig. 1A) and the sample rechromatographed (Fig. 1B). The predicted area resulting from the co-elution and detection of inositol and arginine was 42% of the actual total area for both com-

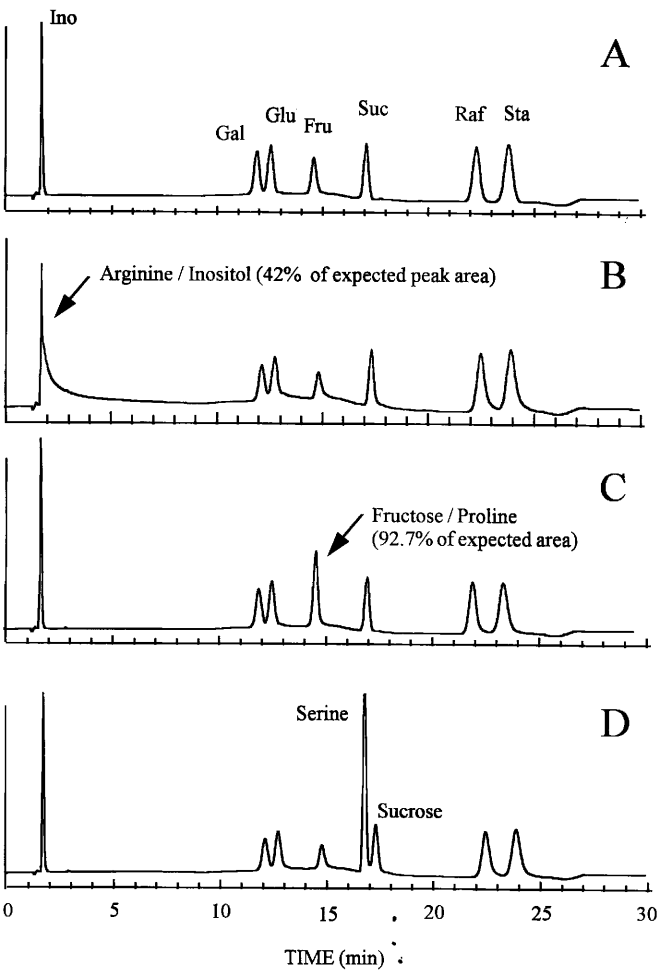

Fig. 1. Sugar standards and interfering amino acids.

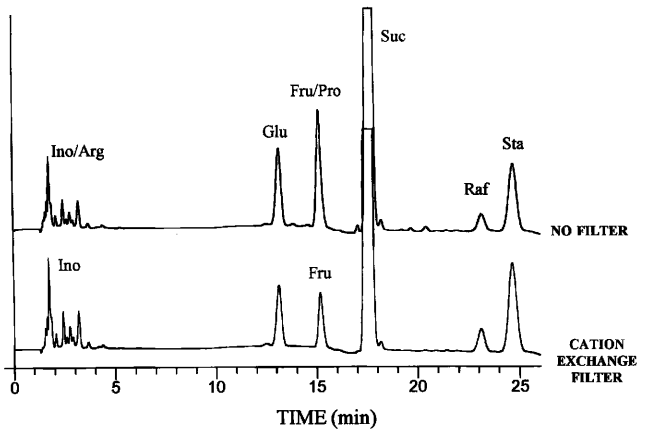

Fig. 2. Removal of amino acids from peanut extracts.

pounds. Therefore, the co-elution of arginine with inositol resulted in a "subtractive" effect because inositol and arginine are detected at the gold electrode by different mechanisms. The addition of proline to the sugar standard directly interfered with the amperometric detection of fructose by co-eluting (Fig. 1C). The resulting response was 92.7% of the expected combined area of the two compounds. Serine eluted very close to the leading edge of sucrose (Fig. 1D). This created a potential error when analyzing peanut sugars because sucrose constitutes about 90% of the total sugars (Oupadissakoon *et al.*, 1980). Serine may affect the determination of sucrose concentration if chromatographic conditions result in co-elution of these two compounds. In addition, if the concentration of amino acids in the extract is sufficiently high, the potential of the detector can be driven to the negative region.

Removal of Amino Acids from the Peanut Extracts. The first approach to remove amino acids from peanut extracts was to use small cation exchange columns. This was an effective procedure, but it increased analysis time by adding the extra step of eluting the sugars and additional time was required for regenerating the columns. Diluting the sample was attempted, but this was not effective for the following reasons. First, after repeated injections, an accumulation of amino acids on the analytical column occurred which reduced retention time of the analytes and eventually led to a deterioration in column performance. Secondly, excessive dilution resulted in loss of the minor constituents in raw peanut meal, including glucose and fructose. A combination of diluting the extract somewhat (optimum concentration of the final extract was 0.1 mM), followed by filtration with a syringe filter instead of columns appeared to be an acceptable alternative. By first diluting the extract, the capacity of the syringe filters was not exceeded. When the sample was filtered, the area counts for inositol increased while glucose and fructose decreased slightly (Fig. 2). Interfering substances on the leading edge of sucrose disappeared, and the response for both raffinose and stachyose improved slightly. These results show that diluting the sample first and filtering the sample through a cation syringe filter is an effective procedure for removing amino acids in the peanut extract.

Conclusions

Separation of sugars in raw peanut extracts on an anion exchange column followed by subsequent detection using an amperometric detector is a very sensitive and useful method. Derivatization is not required, and only a dilute solution of NaOH and water are required for the elution of the analytes. However, interfering substances must be removed for good quantitative results. Cation syringe filters were effective for removing interfering amino acids present in peanut extracts used for sugar analysis. Confirmatory test on the filtrate from the cation filters reacted with ninhydrin showed that interfering amino acids were removed.

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