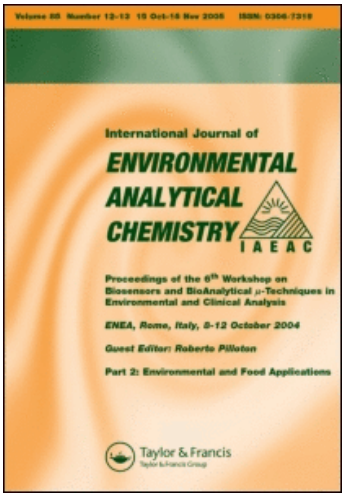

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DETERMINATION OF GLYPHOSATE AS *N*-NITROSO DERIVATIVE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE DETECTION

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Glyphosate (GP) is a non-selective, postemergence herbicide widely used for weed and vegetative control. This paper describes a method for the determination of GP that is based on: a) nitrosation of GP to *N*-nitroso-GP (NGP), b) HPLC separation on an anion-exchange column, c) post column denitrosation of NGP to nitric oxide, and d) chemiluminescence determination of the liberated nitric oxide by a thermal energy analyzer. The method was applied to the determination of GP in water, beer and ale, lentils and beans, and a few cereals. Sample extracts were cleaned-up on mini anion-exchange columns prior to nitrosation. The recoveries of GP added to various samples at 0.01 to 6 μg/g levels ranged between 67–100% with the minimum detection limits ranging between 0.005 to 1 μg/g. The technique will be useful for detection of maximum residue limits (1 to 15 μg/g) of GP in most crops as permitted under Canadian Food and Drugs Regulations.

KEY WORDS: Glyphosate, *N*-(phosphonomethyl)-glycine, chemiluminescence-determination.

INTRODUCTION

Glyphosate (GP) or [*N*-(phosphonomethyl)-glycine] is a non-selective, post emergence herbicide widely used for the control of weeds in the cultivation of many agricultural commodities[1,2]. Since its first introduction by Monsanto Company in 1971, it has become one of the popular herbicides in the market and found wide acceptance among farmers. The two reasons for its popularity seem to be its low mammalian toxicity and its effectiveness against most herbaceous perennial weeds. Upon foliar application, it is quickly absorbed through the leaves and translocated throughout the plant. The major metabolite of GP in plants and mammals seems to be aminomethylphosphonic acid (AMPA).

GP is registered in Canada for both pre-plant and post harvest uses on a number of crops[3]. More recently, pre-harvest application of the herbicide on certain cereals, oilseeds, and pulses (e.g., peas and lentils) has also been approved. The latter application is believed to facilitate harvesting of the crops by drying down the leafy portions of the plants as well as the weeds that might be present at that time. This practice, however, is more liable to leave higher residue levels in the final product for GP is applied close to

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harvest time. To guard against any misuse of the herbicide, Health Canada and Agriculture Canada have set strict regulations as to the maximum permissible residue levels of both GP and AMPA in various food crops. These vary between 2–15 μg/g levels of total GP and AMPA depending on the product. In addition, the guidelines also stipulate the amount and the time of the treatment (e.g., single application at the rate of 0.89 kg/ha GP 7–14 days before harvest)[4]. Further details can be obtained from the Canadian Food and Drug Regulations[3].

Because of the possible health hazard concern, there is a need for reliable analytical methods to closely monitor residue levels of both GP and AMPA in GP-treated crops and vegetables. Such an analysis, however, seems to pose several problems because of the following reasons. First, both these compounds are highly polar and not amenable to extraction with organic solvents from aqueous media. This makes the extraction and preconcentration steps quite lengthy and time consuming. Secondly, neither of them exhibit strong UV absorbance or fluorescence thus making it difficult to develop sensitive HPLC methods for these compounds without derivatization with a suitable chromophore or a fluorophore. For these reasons, most of the methods reported thus far either employ post— or precolumn derivatization prior to HPLC or GC analyses[5]. Despite these difficulties, considerable progress has been made in recent years on the determination of GP and AMPA in various commodities.

Although most of the methods mentioned above work satisfactorily, it was thought that another novel way to determine GP would be to nitrosate it to *N*-nitroso-GP (NGP) (Figure 1) and then analyze the compound by using a thermal energy analyzer (TEA)—a detector that is highly sensitive and specific to *N*-nitroso compounds. In fact, several researchers have already used the *N*-nitroso derivative for TLC and GC determination of GP in water and agricultural products[6–8]. But the possibility of using the TEA for the determination of GP has not been investigated previously. In this paper, we wish to report such a method that has been successfully applied to the determination of GP in water, beer, cereals, and some vegetables.

EXPERIMENTAL

Apparatus

The apparatus used for the HPLC-chemiluminescence determination of NGP consisted of three parts: a) An HPLC assembly, b) a post column reactor to convert NGP to NO, and c) a TEA detector.

a) HPLC assembly A Beckman pump, Model 110B, (Beckman, San Ramon, CA, USA) equipped with an Altex Rheodyne injector (Model 7125, sample loop 50 or 100 μl) was used to deliver the mobile phase. The chromatographic separation was carried out using a Dionex anion-exchange AS4A column (250 mm × 4 mm i.d.) and a Hamilton PRP × 100 guard column (3.6 cm × 4.6 mm; 5 μm) with 0.05 M H_3PO_4 as the mobile phase. The mobile phase flow rate was either 1 or 2 mL/min.

b) Post-column reactor The reactor used for chemical denitrosation of NGP was modified from that described earlier by Havery[9]. These modifications have been described previously by us for the HPLC-chemiluminescence determination of nitrite[10]. In principle, the effluent from the HPLC column is mixed with 10% potassium iodide

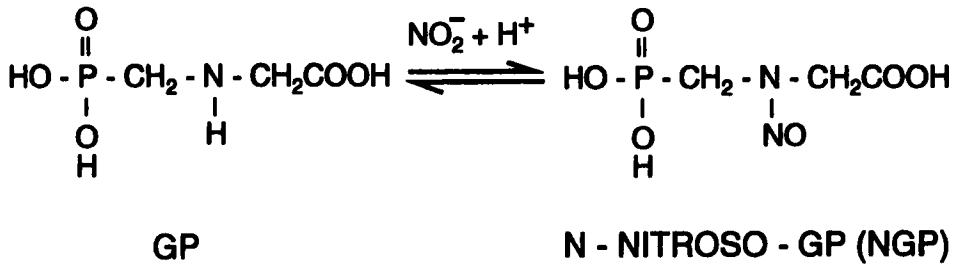

Figure 1 Chemical formula of GP, and the nitrosation reaction showing its conversion to NGP.

solution and a mixture of 10% conc. H_2SO_4 in glacial acetic acid. The mixture is then allowed to flow through a coiled PTFE tubing immersed in a hot (70°C) water bath. In this study, however, instead of using plain 10% KI solution, a mixture of 10% KI solution (100 mL), 0.5 mL sulfamic acid (20 mg/mL), and 1 mL of glacial acetic acid was used. The added sulfamic acid destroyed any nitrite impurity in the KI reagent thus resulting in a lower background noise and, hence, greater sensitivity. A slow stream of Ar carrier gas was introduced into the system just after the post column reactor that carried the generated NO (through several cold traps) into the TEA. The optimum flow rates for various reagents and gases were as follows: the iodide acid mixture, 0.5 mL/min; H_2SO_4 + HOAc, 3 mL/min; and the carrier gas, 30–35 mL/min. Other details (e.g., design of the reactor, cold traps, cleaning and maintenance of the reactor) can be obtained from our previous publication on nitrite[10].

c) Chemiluminescence detection The NO generated by denitrosation of NGP was detected by a TEA detector (Model 502, Thermedics Detection, Chelmsford, MA, USA). Basically, it is a chemiluminescence detector that works on the principle of reaction of ozone with NO to form excited molecules of NO_2* followed by decay of NO_2* to its ground state with subsequent emission of light in the near infra red region (600–3000 nm). The emitted light is amplified and detected using a 1-mv recorder. Further details of the principle and operation of the TEA can be obtained from Fine *et al.*[11,12]. The corrosive acid vapours (acetic acid, H_2SO_4, HI) and moisture are first removed by passing through a series of cold traps because the former may corrode the instrument and the latter interferes with chemiluminescence detection (produces noisy baseline). The TEA reaction chamber pressure was maintained at 0.6–0.7 mm Hg by controlling the carrier gas flow rate as mentioned above under section (b).

Reagents

All reagents were of analytical grade, and all organic solvents used were of glass distilled variety. Glyphosate (GP) was obtained from Monsanto Company (St. Louis, MO, USA). The anion-exchange resin (AG 1-X8, 100–200 mesh, chloride form) was purchased from Bio-Rad Laboratories, Richmond, CA, USA. Water used for the preparation of reagents, standard solutions, and mobile phases was distilled from an all glass apparatus. The concentrations of GP in the stock and working solutions, both in water, were 0.1 mg/mL and 0.01 mg/mL, respectively.

Samples

The samples of foods, vegetables, and beers analyzed in this study were all purchased locally. Many of the samples (e.g., rice, barley, corn, wheat flours) were already in a finely powdered form, and, therefore, were analyzed directly. Other food crops (e.g., lentils, beans) were homogenized using a blender before analysis. Aliquots of the beer samples were first allowed to degas by mixing in an ultrasonic mixer for 30 min before taking an aliquot for analysis.

Method

a) Sample preparation The extent of sample preparation and clean-up required depended on the type of sample analyzed, but all needed some clean-up or preconcentration. For the cereals, a 10 g aliquot was first extracted with 100 mL of water by homogenizing in a Sorval Omni Mixer (DuPont Instruments, Newton, CT, USA) for 5 min, and then the mixture was centrifuged for 15 min at 2000 r.p.m. The supernatant was carefully decanted into an Erlenmeyer flask, and a 25 mL aliquot was taken for the clean-up on an anion-exchange resin column which is described below under subsection (b). The yellow beans were also processed as above.

For the red and green lentils, and whole bean flour, the above extraction method had to be modified because of the formation of viscous and coloured extracts. These samples were extracted with dilute sulfuric acid (3 mL 3 N H_2SO_4 + 97 mL water), the extract centrifuged as above, and the supernatant was partitioned with 100 mL of dichloromethane by gentle shaking in a separatory funnel. The aqueous layer was again centrifuged (to break-up any emulsion), and a 10–20 mL aliquot of the supernatant was used for the ion-exchange clean-up.

b) Anion-exchange clean-up Prior to the actual clean-up, a set of 6 to 8 mini columns were prepared using the resin which had already been allowed to soak overnight (12–16 h) in water. The mini columns were prepared from 20 cm long Pasteur pipettes containing glass wool plug inserts to hold the resin. The actual bed volume of the resin in each column was about 5.5 mm × 3 to 4 cm. Each column was then washed with 25 mL of 1 N NaOH solution (to convert the resin to the hydroxide form) followed by thorough washing with water until the pH of the effluent was close to neutral. The columns were stored in water (in a beaker) until ready to use.

Just before use, a small funnel was attached with a piece of tygon tubing to each mini column. A suitable aliquot of the sample extract was adjusted to pH 10 with 1 N NaOH, filtered if necessary, and poured onto the funnel, small portions at a time, until all of it had gone through the column. Any air bubbles or air gaps inside the funnel stem or the resin bed were broken up with a thin glass rod. At the end, the container was rinsed with 5–10 mL of water and the rinse was passed through the column as above. The column was then successively washed with 2 mL of 0.2 N KOH solution and 3 mL of water, and the washings discarded. GP, if present, was finally eluted from the column by passing 4 mL of 1 N H_2SO_4.

c) Nitrosation The above eluate was mixed with 1.5 mL of freshly prepared $NaNO_2$ solution (10 mg/mL), and the mixture was allowed to stand at room temperature in the dark for 1 hr, with occasional swirling. At the end of nitrosation, the excess nitrite was destroyed by the addition of 1.5 mL of sulfamic acid (20 mg/mL). The solution was made up to 10.0 mL by adding the required amount of water.

A suitable aliquot (0.1 to 1.0 mL) of GP working standard solution was processed as above except that prior to the nitrosation step, a 4 mL aliquot of 1 N H_2SO_4 was also added in order to ensure strongly acidic conditions necessary for efficient nitrosation of GP.

Exactly 5.0 mL aliquots of above solutions (sample or standard) were transferred into separate polyethylene centrifuge tubes (15 mL), and the solutions were adjusted to pH 1 to 1.2 (pH meter) by gradual addition of $Ba(OH)_2$ crystals (using a micro spatula) and mixing in a vortex mixer. This was done slowly and carefully because the final pH attained was found to be very critical (discussed later). The mixtures were then centrifuged for 10–15 min at 2000 r.p.m., supernatants decanted into separate test tubes, and aliquots analyzed by HPLC-TEA. If not analyzed on the same day, the solutions were stored in a refrigerator.

d) HPLC-TEA determination Before analysis, the post column denitrosation apparatus, the TEA, and the HPLC unit were set up as described above. At the beginning of each day, a 25–50 µl aliquot of sodium nitrite solution (1 µg/mL in water, pH 8) was injected at a TEA attenuation setting of 256 to determine if the whole system was working satisfactorily. This should produce a peak height of 10 ± 2 cm depending on the conditions used. Following this, duplicate portions (20–100 µl) of suitable NGP standards or nitrosated sample extracts were injected and analyzed by the HPLC-TEA technique. The concentration of NGP in a sample extract was calculated from its relative response (peak height) to that of an appropriate NGP standard producing a comparable (within ± 50%) response. A standard curve for NGP was occasionally prepared to ensure linearity of the response. For this purpose, 1, 2, 4, 8, and 10 µg aliquots of GP standard were nitrosated, the nitrosated solutions treated with $Ba(OH)_2$, and the final solutions analyzed as above.

RESULTS AND DISCUSSION

As mentioned earlier, several workers have already used nitrosation of GP as the preferred derivatization procedure for the determination of GP in water, plants, and soils[6–8,13]. The reaction is very simple, it proceeds smoothly at room temperature, and gives nearly quantitative yield within 30 min. The derivative (NGP) is also very stable and can be kept at 4°C for at least a week without any noticeable change[13]. Therefore, the procedure should be preferable to others which require more rigorous derivatizations conditions or involve the use of toxic or hazardous chemical reagents[7,14–16]. There is some drawback, however, with this technique. It is not applicable to the determination of AMPA which does not form an *N*-nitroso derivative.

In previous research involving determination of GP as NGP, most workers used GC or TLC methods. Thus far, only Pastore *et al.* have reported an HPLC method for its determinations[8]. These workers used ion chromatography for the chromatographic separation of NGP on a Dionex anion-exchange (AS4A) column, and used either an UV (244 nm) or a conductivity detector for quantitation. In our preliminary studies, we investigated the above column as well as three others (Figures 2 and 3) to determine their suitability or compatibility for our intended purpose. In two of these cases (C_{18} and LC-SAX), NGP gave 2 peaks corresponding most likely to its *syn-* and *anti-* conformers. This agreed with observations made previously by Pastore *et al.*[8] who upon HPLC analysis of NGP on the Dionex AS4A column obtained either a single peak or double peaks depending on the pH of the mobile phase used. The use of 0.01 M $(NH_4)_2$ SO_4,

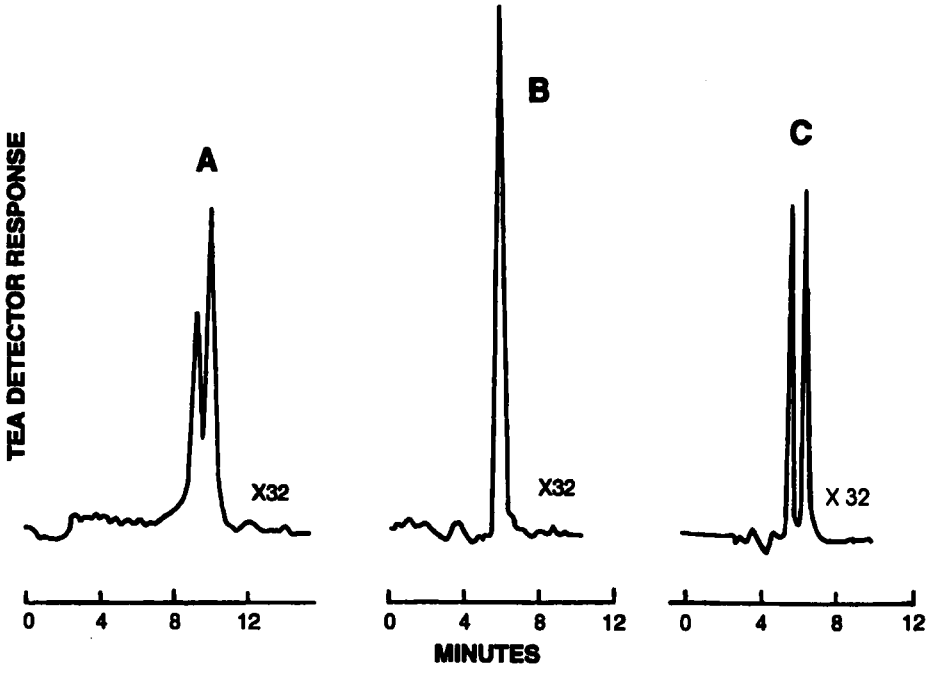

Figure 2 HPLC-TEA determination of NGP on three different columns: (A) 14 ng NGP analyzed on a reversed phase C_{18} column using a mobile phase consisting of 0.05 M KH_2PO_4 (pH 6.0) with 10% methanol and 5 mM tetrabutylammonium hydrogen sulfate as an ion-pair reagent. Flow rate, 1 mL/min. (B) 112 ng NGP analyzed on a Hamilton PRP × 100 column using the above mobile phase adjusted to pH 12. Flow rate, 2 mL/min. (C) 66 ng NGP injected on a Supelcosil LC-SAX column using a mobile phase consisting of 0.05 M K_2HPO_4 (80%) and acetonitrile (20%), pH 6. Flow rate, 2 mL/min.

pH 5.6, as the mobile phase resulted in 2 peaks, whereas the use of 0.01 M $Na_2 SO_4$, pH 12, gave only a single peak. With the other two columns (PRP × 100 and Dionex AS4A), we obtained only single peaks under the conditions (Figures 2 and 3) used. The strongly acidic mobile phase (0.05 M H_3PO_4) used with the Dionex AS4A column was most likely responsible for the differences in results from that obtained by the above researchers[8] who observed two peaks using the weakly acidic (pH 5.6) mobile phase. The single peak, however, made quantitation much easier.

Nitrosation of GP

Previous studies have shown that due to its weakly basic nature, GP nitrosates rapidly and quantitatively under acidic conditions[13]. In preliminary experiments the optimum conditions for its nitrosation was determined by varying the different parameters, namely, nitrite concentration, pH of the reaction medium, and the time. All reactions were carried out at room temperature. For low levels of GP (1 to 25 μg in 1 mL), incubation with 2 mg $NaNO_2$ and 4 mL 1 N H_2SO_4 for 30 min gave the maximum yield of NGP. However, slightly higher amounts (15 mg) of $NaNO_2$ have been incorporated in the protocol to compensate for any nitrite-scavenging materials that might be present in sample extracts. Increasing the incubation period to 1 hr or increasing the reaction

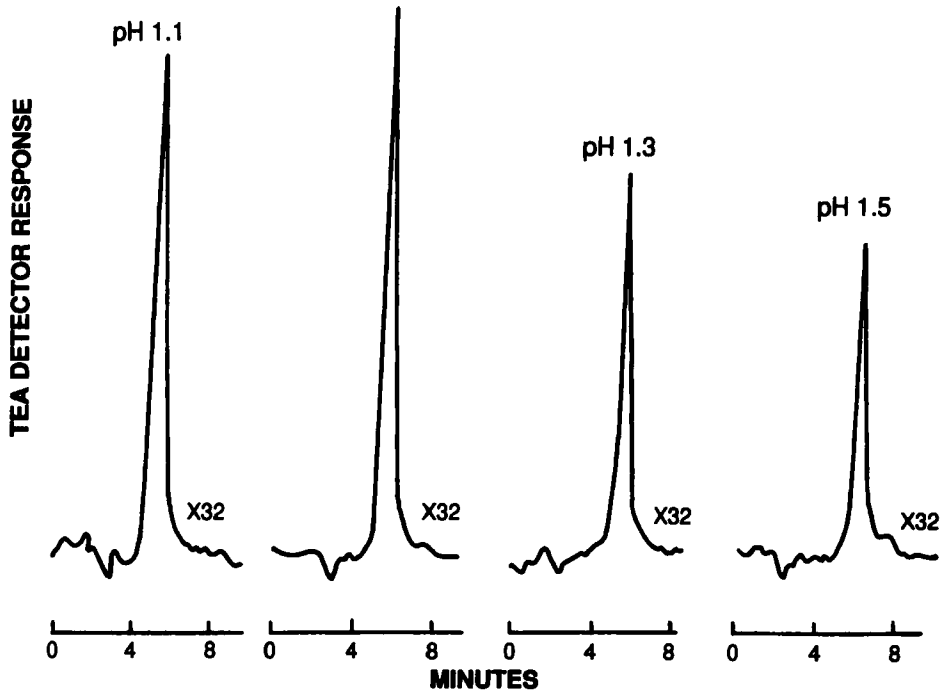

Figure 3 Relationship between the final pH obtained after neutralization with $Ba(OH)_2$ and the peak height of NGP. HPLC column: Dionex AS4A anion-exchange column with a Hamilton PRP × 100 guard column as mentioned under EXPERIMENTAL. Mobile phase, 0.05 M H_3O_4; flow rate, 2 mL/min. About 30 ng NGP was injected in each case.

temperature to 50°C did not increase the yield any further. Therefore, the conditions chosen for nitrosation of GP should give maximum yield of NGP under most circumstances.

Chromatography

Two major problems were encountered during HPLC analysis of nitrosated GP standard and sample extracts. One of them was due to the presence of excess acid in the reaction mixture that interfered with the chromatography especially when the Dionex AS4A column was used. Although the excess acid could be neutralized by careful addition of dilute NaOH or KOH, this resulted in an excess concentration of salt which forced very early elution of the NGP peak. This problem was resolved by neutralizing the excess sulfuric acid with the careful addition of $Ba(OH)_2$ crystals. The SO_4^{-2} ions were precipitated as insoluble $BaSO_4$ without increasing the salt concentration in the reaction mixture. The final pH attained after neutralization with $Ba(OH)_2$, however, seemed to be critical for the yield (peak height) of NGP. This is illustrated in Figure 3 which shows a final pH between 1.1 to 1.2 to be the desired range. Above pH 1.5, the peak height dropped sharply. It is possible that at a higher pH, the phosphate group in NGP forms an insoluble salt, thus reducing its concentration in the reaction mixture.

The other problem, mentioned above, had to do with the appearance of an interfering peak very close to the NGP peak in blank as well as most sample extracts. This always happened when a C_{18} or a PRP × 100 column was used (Figure 4). After failing to determine the cause of this interference or to resolve the problem, these two columns were no longer used in the study. Similar problem was also encountered with the Dionex AS4A column when either 0.01 M $(NH_4)_2 SO_4$ (pH 5.6) or 0.01 M $Na_2 SO_4$ (pH 12) was used as the mobile phase. Pastore *et al.*[8], on the other hand, did not observe any such interfering peak even though they used the same column and same mobile phases. These researchers, however, did not apply their method for the determination of GP in foods or environmental samples. They also used different types (UV and conductivity) of detectors. It is possible that the interfering compound gives a positive response in the TEA system but not in the others. Finally, during experimentation with different mobile phases it was observed by chance that a mobile phase consisting of 0.05 M H_3PO_4 gave none or negligible interfering peak when used in combination with the Dionex AS4A column and the TEA detector. Hence, this combination was used in subsequent studies.

Applications

The method was used to determine its suitability for the determination of GP in water, beer, and several agricultural products listed in Table 1. Some kinds of preconcentration or clean-up of sample extracts were, however, required prior to derivatization and HPLC-TEA analysis. Because of its extremely polar nature, ion-exchange clean-up seems to be the method of choice and has been successfully used by previous researchers[13,17,18]. Although some workers have used clean-ups on both cation-exchange and anion-exchange resin columns, the use of only one— the anion-exchange resin clean-up— was found to be adequate in the study. This was possible mainly because of the extremely high specificity of the TEA detector.

The above clean-up served two purposes. First, it helped in removing some of the unwanted materials from sample extracts that might have interfered with the nitrosation

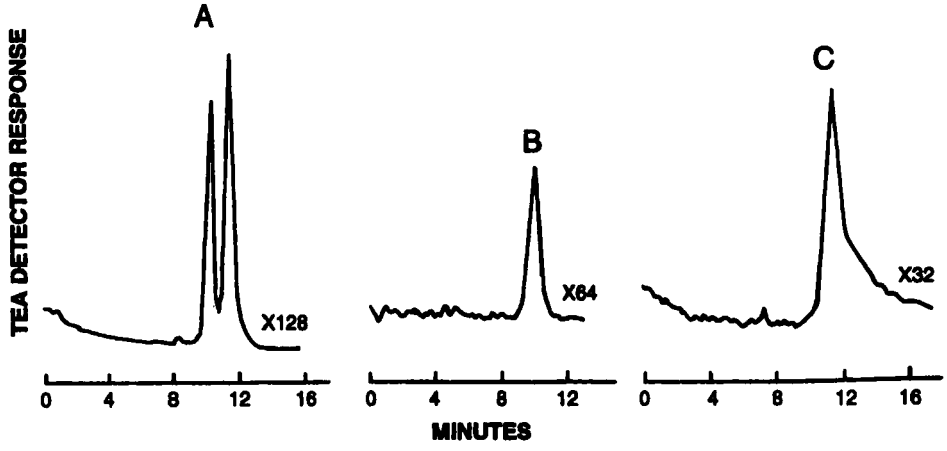

Figure 4 HPLC-TEA chromatograms using the reversed phase C_{18} column: (A) 14 ng NGP standard, (B) reagent blank, and (C) 20 ng NPRO standard. Conditions were the same as in Figure 2(A).

Table 1 Percentage recoveries of GP from various fortified materials[a].

Samples analyzed	*Fortification level (μg/g)*	*% Recoveries*
Water		
A	0.1	95
A	2.5	83
B	0.1	98
B	0.025	85
C	0.01	92
C	0.025	100
C	0.05	83
D	0.01	70
D	1.2	100
Beer and Ale		
A	0.1	71
A	1.2	86
B	0.25	88
C	0.25	100
D	0.25	79
Cereals		
Rice flour	0.5	76
Rice flour	1.0	72
Corn	0.5	100
Barley	0.5	67
Barley	2.5	91
Rye	0.5	100
Green lentils	6.0	83
	3.0	97
Fresh beans	2.5	87

[a]Unfortified samples were either negative for GP or contained negligible levels. Blank values were subtracted before calculating recoveries.

or TEA determination step. Secondly, it also helped in preconcentration of the analyte. The maximum preconcentration was achieved for water because up to a 100 mL aliquot could be passed through the resin column and the adsorbed GP be eluted in 4 mL of eluate. In most cases, however, 50 mL aliquots of water were used for the analysis. For beer and ale, a 25 mL aliquot was used for the clean-up corresponding to about a 6 fold concentration. For the cereals, beans, and the lentils, an extract equivalent to 2 to 2.5 g of the original material was passed through the resin column. In these cases, the preconcentration factor was about 5 to 6 fold in terms of the extract volume passed through the column.

The method worked well with all the samples analyzed thus far. The recoveries of GP added to various substrates at 0.01 to 6 μg/g levels ranged between 67–100% (Table 1) which are comparable to results obtained using other methods[4,13,19,20]. Both the precision of the HPLC-TEA determinative step as well as that of the overall method were found to be highly satisfactory. The former was always within ± 5% and the latter in most cases was within ± 10%. The minimum detection limit (> 3 × noise level) of the method in various commodities depended on the sample size used for the analysis. For water, it was estimated to be about 0.005 μg/g; for beer and ale, about 0.1 μg/g; and for the other

samples it was between 0.5–1 μg/g. Since the maximum residue limits for GP in various crops are in the range of 1 to 15 μg/g levels, the method should be useful for monitoring violative residue levels of GP in various agricultural products. Some typical chromatograms obtained from the analysis of above products are shown in Figures 6–7.

There is a possibility that a secondary amino acids such as L-proline, which is a naturally occurring amino acids in foods, can form an *N*-nitroso derivative and, if not well resolved from the NGP peak upon chromatography on the HPLC column, may give a false-positive result. To test this possibility, NPRO standard was analysed by this technique using the HPLC columns mentioned above. It eluted very close to NGP on the C_{18} column (Figure 4), but did not elute at all (gave a negative result) on the Dionex AS4A column (not shown). It is highly unlikely, therefore, NPRO would interfere with the analysis under the conditions employed.

In summary, we have developed a new HPLC-TEA method for the determination of GP that is quite sensitive and highly specific. Preliminary results suggest that the method is applicable to the determination of GP in a variety of products. The technique can be used as such or for confirming results obtained by another method. It is hoped that the method will be useful for both research and monitoring purposes.

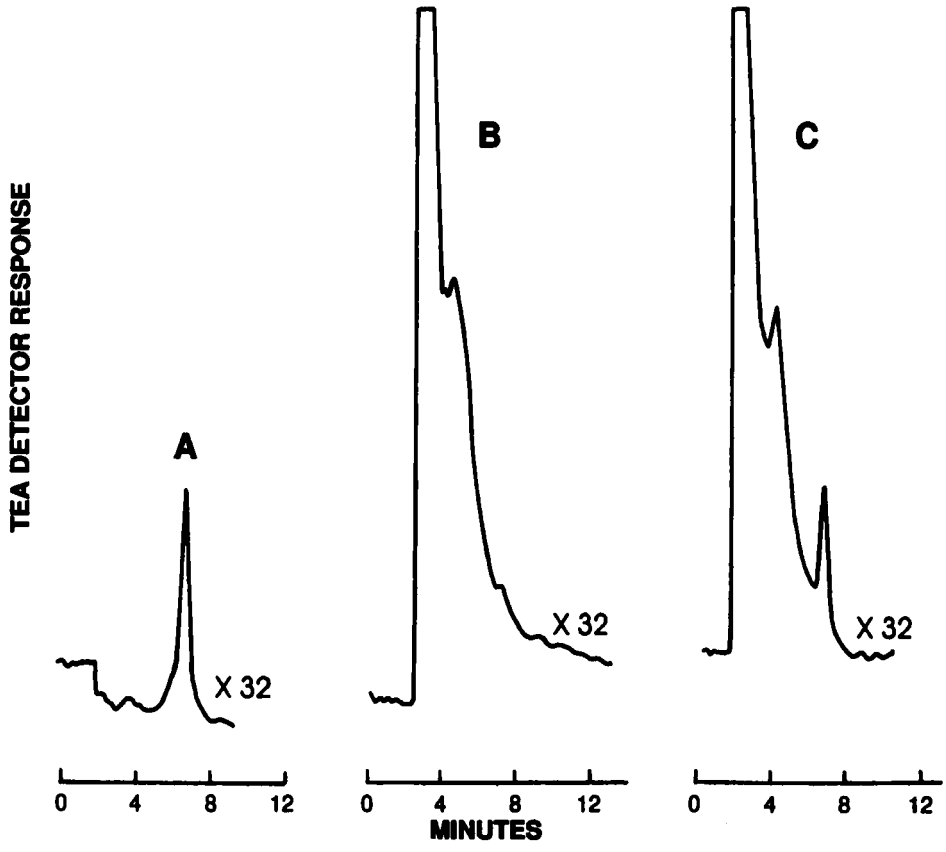

Figure 5 HPLC-TEA analysis of beer for GP. (A) 15 ng NGP standard, (B) nitrosated beer extract, unspiked, (C) the same spiked with 0.25 μg/g level of GP. Conditions were the same as in Figure 3.

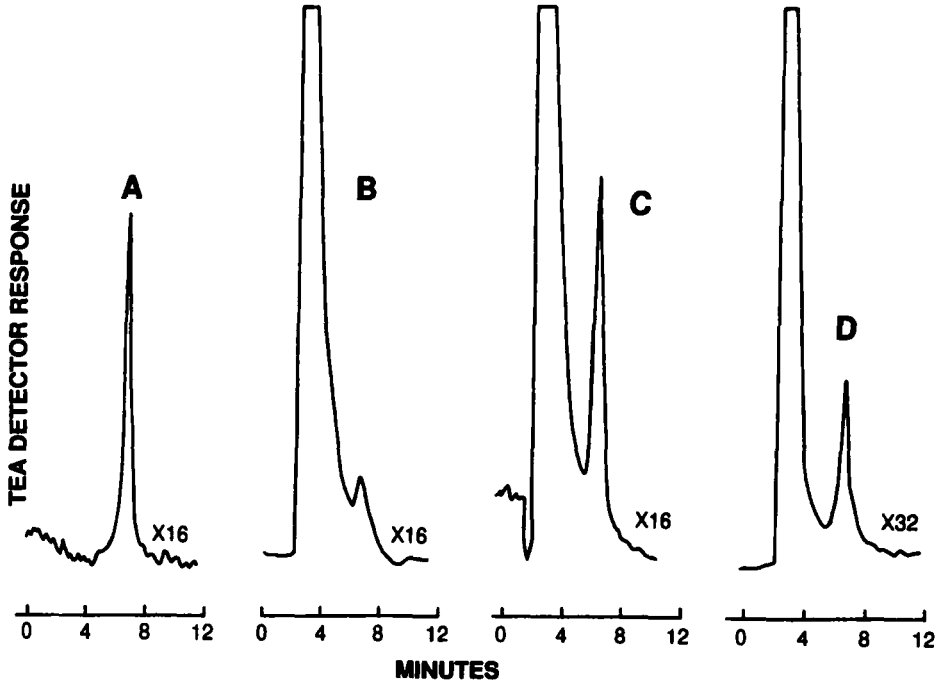

Figure 6 Chromatograms obtained from the HPLC-TEA analysis of some additional samples. (A) 15 ng NGP standard, (B) nitrosated extract of unspiked yellow beans, (C) nitrosated extract of the same spiked with 2.5 μg/g GP, and (D) nitrosated extract of green lentils spiked with 3 μg/g GP (that of unspiked green lentils not shown). Conditions were the same as in Figure 3.

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