Research Article

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Liquid chromatographic determination of glyphosate and aminomethylphosphonic acid residues in rapeseed with MS/MS detection or derivatization/fluorescence detection

DOI: 10.1515/chem-2015-0107
received December 8, 2014; accepted April 1, 2015.

Abstract: Glyphosate and AMPA determinations in rapeseed extracts by (a) liquid chromatography with triple quadrupole MS detection (LC-MS/MS), or (b) liquid chromatography with post-column OPA derivatization and fluorescence detection (LC-FLD) were developed. Mean recoveries for glyphosate and AMPA were (a) 88.8–95.0% and 82.1–86.1%, and (b) 70.8–74.1% and 62.4–72.6%. RSD were below (a) 11% and (b) 22%. Correlation coefficients were above 0.997 for both methods. LOD were 0.01 mg kg\(^{-1}\) for (a) and 0.05 mg kg\(^{-1}\) for (b). Both methods are simple and efficient for routine analysis of glyphosate and AMPA in a fatty matrix.

Keywords: glyphosate, AMPA, rapeseed, liquid chromatography, mass spectrometry

1 Introduction

Glyphosate (N-phosphonomethyl glycine) is a pesticide widely used in agriculture, horticulture, and silviculture as well as around homes and gardens [1]. Introduced by Monsanto in the early 1970s, it is a broad spectrum, nonselective, post-emergence herbicide [2] that inhibits plants’ shikimic acid pathway [3,4]. In some countries glyphosate is also used before harvest to control weeds and to speed rapeseed maturation (dessication) [5].

Glyphosate absorption is only effective when sprayed on foliage and it is immobilized in soils [6]. It is quickly biodegraded to aminomethylphosphonic acid (AMPA), which is mineralized by bacteria [7].

Glyphosate is considered to have low mammalian toxicity and no adverse effects in humans [8,9]. However, this is controversial since several studies suggest toxic effects [10-14]. Maximum glyphosate residue levels (MRLs) are around 0.1 mg kg\(^{-1}\) for most plant products in the European Union, and 30 mg kg\(^{-1}\) for cereal grains (Codex Alimentarius), depending on the crop, country, and organization.

The determination of residual pesticides in a rapeseed fatty matrix (98–99% triglycerides) is very challenging [15,16]. Glyphosate and AMPA are difficult herbicides in trace analysis. They have low molecular weight, low volatility, thermal lability, and good water solubility. These properties cause problems in extraction, purification and determination.

Several analytical procedures have been developed based on solid-phase extraction [17], ion-exchange chromatography [18], or matrix soil phase dispersion (MSPD) [19]. Most published methods involve liquid extraction of soil followed by cleanup [20-23]. There have been few reported methods for determining glyphosate in fatty matrices [21,24,25], and none in rape. A simple, sensitive, selective, and accurate method is required.

Glyphosate has usually been quantified by chromatographic methods. High-performance liquid chromatography with fluorescence detection (HPLC-FLD) or ultraviolet detection (HPLC-UV) [23,26,27] have an excellent track record in its determination. Other techniques include capillary electrophoresis [28,29], ion chromatography with conductivity [30] or fluorescence [31], gas chromatography [32,33], immunoassays [34,35], nuclear magnetic resonance [36], and integrated pulse amperometry [37].

All these methods require a derivatization, depending on the procedure and detection technique. Derivatization with p-toluenesulfonyl chloride [12], p-nitrobenzoyl...
chloride [38], 9-fluorenylmethyl chloroformate (FMOC-Cl) [39-41], (+)-1-(9-fluorenyl)ethyl chloroformate [42], trimethyl orthoacetate, or N-methyl-N-(tert-butylmethylsilyl)trifluoroacetamide [43], makes the determination both tedious and time-consuming.

Tandem mass spectrometry (MS/MS) is currently the preferred technique for polar pesticide residue analysis due to its excellent sensitivity and selectivity. LC-MS/MS has been used to detect glyphosate and AMPA [44,45] and glyphosate residues have been determined in a variety of environmental and food matrices after derivatization with FMOC [22,35,44-48]. Over the last few years new methods have been reported which avoid derivatization [20,21,48].

The rapeseed trade is economically important, and the presence of glyphosate residue is an important quality indicator. Thus the seed must be analyzed. The goal of this study was to develop procedures for glyphosate and aminomethylphosphonic acid (AMPA) residue determinations in rapeseed, and to compare liquid chromatography with detection by a triple quadrupole mass spectrometer without derivatization and detection by post-column OPA derivatization and fluorescence (LC-FLD).

2 Experimental

2.1 Chemicals and standards

Glyphosate, AMPA and isotope-labeled glyphosate ($^{13}$C$_2$N) reference standards (purity > 98.7%) were purchased from Sigma-Aldrich (Poznan, Poland). Orthophosphoric acid, boric acid, potassium dihydrogen phosphate, sodium hypochlorite, sodium hydroxide, sodium chloride, potassium hydroxide, o-phthalaldehyde, 2-mercaptoethanol, formic acid, acetic acid, ammonium formate and ammonium acetate were purchased from Merck (Darmstadt, Germany). HPLC and LC-MS grade acetonitrile and methanol were purchased from POCh (Gliwice, Poland). LC-grade water (18 MΩ cm) was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA).

Standard stock solutions were prepared by dissolving approximately 50 mg glyphosate and AMPA powders in 100 mL of water for a final concentration of approximately 500 mg L$^{-1}$. The working standard solutions of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 10.0 mg L$^{-1}$ (10 mL each) for chromatographic analysis and sample fortification were prepared by dilution with water.

2.2 Analytical methodology

2.2.1 Sample preparation

A homogenized 5.0 g rapeseed sample and 10 mL of deionized water containing 0.1% formic acid were transferred to a 15 mL polypropylene centrifuge tube, capped, shaken, and centrifuged (5000 rpm) for 15 and 10 minutes. 2 mL were passed through preconditioned (2 mL of methanol then 5 mL of water) C-18 cartridges (500 mg C18 Supelco Discovery, Supelco), and filtered into a vial for LC-FLD analysis.

For the LC-MS/MS analysis 50 µL of 10 µg mL$^{-1}$ $^{13}$C$_2$N-glyphosate internal standard was added prior to homogenization, solvent addition, shaking, and centrifugation as above. The supernatant was decanted and directly injected into the LC/MS/MS system.

2.2.1.1 Preparation of spiked sample

A blank sample was spiked with standard pesticide solution and equilibrated for 2 h. It was then prepared as above.

2.2.2 Instrumentation

2.2.2.1 Analysis by LC-FLD

A Waters Alliance 2695 (Waters, Milford, MA, USA) HPLC System with a Waters 2475 fluorescence detector, two step post-column derivatization system, controlled by Waters Empower 2 Software was employed.

Separation was performed isocratically on a Supelcosil LC-SCX column (250 × 4.6 mm, 5 µm, Supelco) at 40°C and 0.4 mL min$^{-1}$. The mobile phase contained 0.7 g potassium dihydrogen phosphate per liter of water, adjusted to pH 2 with orthophosphoric acid. After separation the column was regenerated with diluted 0.1 M KOH and re-equilibrated with eluent. Mobile phases were vacuum degassed before use. Oxidizing solution was prepared by adding 100 µL of 5% sodium hypochlorite solution to 1 L of water. Fluorogenic solution was prepared by dissolving 25 g boric acid and 10 g NaOH in 1 L of water and adding 2 mL 2-mercaptoethanol and 1 g o-phthalaldehyde dissolved in 10 mL methanol. The oxidant flow rate was 0.2 mL min$^{-1}$, and the fluorogen, 0.3 mL min$^{-1}$. The first reaction coil was at 40°C and the second at ambient temperature. All solutions were stored in dark bottles. The volume injected into the LC-FLD system was 10 µL. The fluorescence detector excitation/emission wavelengths were 330/465 nm.
2.2.2.2 Analysis by LC-MS/MS

Chromatographic conditions
An Eksigent Ultra LC-100 (Eksigent Technologies, Dublin, CA, USA) liquid chromatography system was operated at 0.4 mL min\(^{-1}\) without split using a Zorbax Eclipse Plus C18 Rapid Resolution HP (Agilent Technologies) analytical column (100 × 2.1 mm, 1.8 µm) at 60°C. The injected volume was 10 µL. The binary mobile phase consisted of 1% acetic acid in water (phase A) and 1% acetic acid in methanol (phase B). The initial composition of 95% A and 5% B (v/v) was held for 1.5 minute, followed by linear ramping to 95% B over 2.5 minutes. After ramping the mobile phase was returned to the initial composition over 1 minute and was held for 3 minutes. The total chromatographic run time was 8.0 minutes.

Mass spectrometry conditions
Mass spectrometric analysis was done on an MS/MS 6500 QTRAP (AB Sciex Instruments, Foster City, CA) with electrospray ionization source operating in negative ionization mode, with ion spray voltage (IS): -4500 V; curtain gas: 35 psi; nebulizer gas (GS1): 60 psi; auxiliary gas (GS2): 70 psi; source temperature: 650°C. Nitrogen was the nebulizer and collision gas. Optimization of the compounds was performed by injecting individual standard solutions directly into the source. The most intense MRM transition was selected for quantitation. AB Sciex Analyst software 1.6.2 was used for data acquisition and processing.

2.3 Method validation

Validation was carried out using glyphosate-free samples according to SANCO Document 12495/2011 [50]. The matrix effect, linearity, limits of detection (LOD) and quantitation (LOQ), recovery and precision were evaluated.

Accuracy and precision were evaluated by recovery studies. Calibration curves were obtained from matrix-matched multi-level calibration solutions. Calibration standards were prepared by spiking a blank rape matrix (three matrix concentration ranges) to produce final concentrations of 0.05, 0.10, 0.50, 1.0, and 2.0 mg kg\(^{-1}\) for LC-FLD and 0.01, 0.05, 0.10, 0.50, and 2.0 mg kg\(^{-1}\) for LC-MS/MS. Linearity was determined from the coefficients of determination (R\(^2\)). The precision was expressed as the relative standard deviation (RSD). Accuracy was evaluated by comparing the measured and true values. The LOQs were defined as the minimum concentration that can be quantified with acceptable accuracy and precision. LOD were concentrations giving signal-to-noise ratio (S/N) of 3.

3 Results and discussion

HPLC analysis of rapeseed presents two distinct challenges. The first is the separation itself. Rape contains a complex mixture of very nonpolar compounds (fatty acids, triglycerides, waxes, etc.). The second challenge is detection. Our compounds of interest have no UV chromophore, rendering traditional HPLC-UV detectors unusable.

3.1 LC-FDL determination

Cation-exchange liquid chromatography of glyphosate and AMPA is well documented [31,51-53]. An LC-SCX cation-exchange column with strongly acidic propylsulfonic acid groups was used for cation separation. They were detected by a fluorescence detector following a two stage post-column reaction. Glyphosate is first oxidized by hypochlorite to a primary amine – glycine. Then, in the second step, glycine and AMPA react with o-pthalaldehyde in the presence of 2-mercaptoethanol to produce highly fluorescent isoindoles.

To minimize band spreading, it was important to keep the time (and volume) between column and detector as low as possible. The effects of reagent flow rate, NaClO concentration, reaction time and temperature were examined. 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 mL min\(^{-1}\) sodium hypochlorite flows were examined; 0.2 mL min\(^{-1}\) was optimal. Oxidizing solution was varied by the addition of 25, 50, 100, 200, 300, 400 or 500 µL of 5% sodium hypochlorite per liter of water. Fig. 1 illustrates the influence of the NaClO concentration on peak areas. Maximum glyphosate response was obtained with 100 µL. At low hypochlorite concentrations glyphosate response was reduced due to incomplete oxidation. AMPA was reactive towards hypochlorite, and its response was decreased by excess hypochlorite (Fig. 1). The hypochlorite concentration slowly decreased over time yet an increase in glyphosate and AMPA peak areas was observed. Similar results have been reported [31,54].

Temperature effects (20–80°C) on the oxidation step were subsequently evaluated. The best glyphosate and AMPA responses occurred at 40°C, at which the reaction time was a minimum (< 1 min.).

Patias and co-workers performed the second stage with thiofluor [31]. In this work, 2-mercaptoethanol used instead giving significantly higher signals. The OPA reagent at 0.3 mL min\(^{-1}\) and ambient temperature gave maximum glyphosate and AMPA responses. To minimize OPA loss by air oxidation the reservoir was maintained under argon.
3.2 Chromatography and mass spectrometry

Tandem mass spectrometry (MS/MS) based on electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) can be a fast method for polar compound determination. Glyphosate and AMPA can be ionized in positive or negative modes through proton addition or loss [20,24,55].

For MS/MS optimization, 1 µg mL⁻¹ glyphosate and AMPA solutions were used to select the three most prominent product ion transitions. The most abundant fragment ions and the ionization parameters for each transition were determined. Full-scan chromatograms indicated compound retention. Then direct sample injection was used to optimize the ion source temperature, ion spray voltage, curtain gas, nebulizer gas, auxiliary gas and collision gas.

Optimized MRM transition parameters: declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) for each of the compounds in negative ion mode are presented in Table 1. The best sensitivity was in “scheduled MRM mode” with a time window of 60 seconds.

Many columns have been tested, including reverse phase [56,57], anion exchange [58], multimode (reverse phase plus weak anion exchange) [59] and HILIC column (hydrophilic interaction liquid chromatography) [21,25,49,55]. Each has worked for a specific matrix; other matrices may require different conditions.

This work used a highly endcapped C18 column which gives poor but sufficient glyphosate and AMPA retention (1.05 and 1.20 minutes). This did not lead to lower detection sensitivity. Thus the direct determination of glyphosate and AMPA is feasible. The column was stable under these conditions; no peak shape degradation or retention loss was observed after approximately 300 runs.

Different mobile phases including water, acetonitrile, and methanol, with addition of acetic acid, formic acid, ammonium formate, or ammonium acetate were compared. Acetonitrile in the mobile phase caused poor retention. Water:methanol (95:5) increased retention times to 1.05 and 1.20 minutes, but gave broad and ragged peaks. Ammonium acetate and ammonium formate additions caused broad peaks. Acetic acid was crucial. Isocratic elution with water:methanol (95:5), both with 0.1% acetic acid gave the best signal to noise ratio, sensitivity and retention time. 10 µL injections gave the highest sensitivity without negatively affecting the peak shape.

3.3. Sample preparation and matrix effect

Since glyphosate and AMPA show high polarity and poor volatility yet easily dissolve in organic solvents such as acetone, hexane, toluene etc. [60] modified aqueous extractants are widely applied. Glyphosate and AMPA can be extracted with borate buffer [23], water: dichloromethane [24], water-methanol-dichloromethane [22], water [55] or KOH solution [48]. Ten milliliters of the following were tested: methanol, acetonitrile, water, tetrahydrofuran and solvent mixtures (Table 2). The best GLY and AMPA recoveries were when water was used (82.0 and 75.2% respectively). As might be expected, acidification improved the results; 0.1% formic acid in water was used.

Targeted compounds are generally present at very low concentrations and many interfering components can be co-extracted, so they must be effectively removed. Due to extensive interference, rice, maize and soybean have needed purification before analysis. Different types

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**Table 1:** Optimised MRM transitions by direct ESI source infusion.

| Analyte     | MRM transition (m/z) | DP (V) | CE (eV) | CPX (V) |
|-------------|----------------------|--------|---------|---------|
| AMPA        | 110 > 81             | -55    | -18     | -5      |
|             | 110 > 79             | -55    | -34     | -3      |
|             | 110 > 63             | -55    | -24     | -9      |
| Glyphosate  | 168 > 150            | -45    | -14     | -9      |
|             | 168 > 124            | -45    | -16     | -7      |
|             | 168 > 63             | -45    | -32     | -3      |
| 13C 15N-glyphosate (ISTD) | 171 > 63 | -45    | -30     | -5      |
of functionalized copolymer SPE cartridges such as CAX, SAX, SCX, Oasis HLB, Oasis MAX, or traditional C18, C8 and silica phases have been used [23,48,60], but there are no reports concerning rape seed purification.

The main problem in GLY and AMPA extraction from rapeseed is the presence of other water-soluble components (Fig. 2a). The matrix strongly influences the SPE extraction efficiency. Two categories of sorbents: the silica-based C18, and the polymer-based SDB-1 types, were examined. C18 gave effective analyte baseline separation along with lower limits of detection, despite impurities remaining (Fig. 2b).

A matrix effect ($M_{\text{eff}}$) causes an analyte measurement to vary due to undetected sample components [61]. To evaluate $M_{\text{eff}}$, two sets of standard solutions containing glyphosate and AMPA at five concentration levels (0.05, 0.10, 0.50, 1.0, 2.0 mg kg$^{-1}$, for LC-FLD; and 0.01, 0.05, 0.10, 0.50, 2.0 mg kg$^{-1}$ for LC-MS/MS) were prepared. The first series was obtained by diluting the working standards in solvent (triplicate). The second was prepared by diluting the same working standards in matrix extracts (triplicate) obtained from pesticide-free samples. LC-MS/MS chromatograms of glyphosate and AMPA in solvent and matrix are presented in Fig. 3. Matrix effects were assessed by comparing the slopes of matrix-matched calibration curves with the slopes of solvent-only calibration curves [50]. The matrix effect percentage ($\%M_{\text{eff}}$) for each analyte is given by (Table 3):

$$\%M_{\text{eff}} = 100\% \left(1 - \frac{\text{slope}_{\text{matrix}}}{\text{slope}_{\text{solvent}}}\right)$$

$\%M_{\text{eff}}$ between ± 20% are not significant, because this variability is close to the RSD [61]. Thus solvent-matched calibration standards can be used for glyphosate quantitation by LC-MS/MS (~20% $M_{\text{eff}}$). In other cases matrix-matched calibration standards should be used.

### 3.4 Method validation

The method was validated in rapeseed as described in 2.2.1, based on European Union SANCO guidelines [50]. Linearity, evaluated through analysis of five standard solutions (0.01–2.0 µg mL$^{-1}$, five replicates), was satisfactory with correlation coefficients ≥ 0.995 (Table 3). Method specificity was evaluated by analyzing procedure blanks, processed blanks, and processed blanks spiked at the lowest concentration level tested.

Accuracy (percentage recoveries) and precision (% RSD) were estimated by recovery from rapeseed

| Extraction solvent                  | Recovery (%) | glyphosate | AMPA |
|-------------------------------------|--------------|-----------|------|
| water : dichloromethane (1:1; v/v) | 58.6         | 53.7      |      |
| water : dichloromethane (2:1; v/v) | 64.8         | 59.4      |      |
| borate buffer (pH=9)                | 30.5         | 28.0      |      |
| KOH solution (0.5 M)                 | 26.7         | 24.5      |      |
| water : methanol : dichloromethane  | 71.2         | 65.3      |      |
| (2:2:1; v/v/v)                       |              |           |      |
| water                               | 82.0         | 75.2      |      |
| methanol                            | 68.2         | 62.5      |      |
| acetonitrile                        | 58.4         | 48.7      |      |
| tetrahydrofurane                    | 32.5         | 28.7      |      |
| water : methanol (1:1; v/v)         | 75.6         | 69.3      |      |
| water (with addition of 1% formic acid) | 88.1     | 80.7      |      |
| water (with addition of 1% HCl)     | 83.3         | 76.3      |      |
| water (with addition of 0.1% formic acid) | 93.4     | 85.6      |      |
| water (with addition of 0.1% HCl)   | 88.3         | 80.9      |      |

Figure 2: LC-FLD chromatograms of glyphosate and AMPA for rape spike at 0.5 mg kg$^{-1}$ (a) without clean-up and (b) after SPE C18 clean-up.
matrices at five fortification levels (0.05, 0.10, 0.50, 1.0, 2.0 mg kg\(^{-1}\) for LC-FLD and 0.01, 0.05, 0.10, 0.50, 2.0 mg kg\(^{-1}\) for LC-MS/MS-), each analyzed in quintuplicate. Accuracy was satisfactory for glyphosate and AMPA by both methods, with recoveries above 64.2% for AMPA/LC-FLD, and above 88.8% for glyphosate/LC-MS/MS. Precision was also satisfactory, with RSDs below 15–20% (Table 3). The inferior accuracy and precision of LC-FLD may be due to the additional clean up.

Secondly, accuracy was assessed by analysis of a sample (EUPC-SRM-8) from the 2013 European Union Reference Laboratory Requiring Single Residue Method (Stuttgart, Germany). This includes 0.34 mg kg\(^{-1}\) glyphosate (RSD 6.5%) – a level which violates the standards. AMPA was not present. Analysis by LC-MS/MS gave a concentration of 0.38 mg kg\(^{-1}\) (Fig. 3e). The back-calculated z-score for this analysis is 0.47. These results indicate that the method is precise and accurate.

| Analyte | Spiked level (mg kg\(^{-1}\)) | Recovery (%) | RSD (%) | LOQ (mg kg\(^{-1}\)) | LOD (mg kg\(^{-1}\)) | Linearity \((R^2)\) (in matrix) | M\(_{\text{eff}}\) (%) |
|---------|-------------------------------|--------------|---------|-----------------------|-----------------------|--------------------------------|------------------|
| AMPA    | 0.05                          | 64.2         | 21.1    | 0.05                  | 0.03                  | 0.9975                         | 45               |
|         | 0.5                           | 69.5         | 17.5    |                       |                       |                                |                  |
|         | 2.0                           | 72.6         | 16.9    |                       |                       |                                |                  |
| Glyphosate | 0.05                          | 70.8         | 14.7    |                       |                       |                                |                  |
|         | 0.5                           | 73.0         | 13.4    | 0.05                  | 0.02                  | 0.9982                         | 32               |
|         | 2.0                           | 74.1         | 12.8    |                       |                       |                                |                  |
| AMPA    | 0.01                          | 82.1         | 10.5    |                       |                       |                                |                  |
|         | 0.1                           | 83.1         | 10.2    | 0.01                  | 0.005                 | 0.9992                         | 29               |
|         | 2.0                           | 86.1         | 8.8     |                       |                       |                                |                  |
| Glyphosate | 0.01                          | 88.8         | 9.2     |                       |                       |                                |                  |
|         | 0.1                           | 92.4         | 7.0     | 0.01                  | 0.005                 | 0.9995                         | 18               |
|         | 2.0                           | 95.0         | 6.9     |                       |                       |                                |                  |
The LOQs, defined as the minimum analyte concentration that can be quantified with acceptable accuracy and precision [50] were (for both glyphosate and AMPA) 0.05 and 0.01 mg kg⁻¹ for the LC-FLD and LC-MS/MS techniques.

The LODs were estimated for a signal to noise ratio of 3 from chromatograms of samples spiked at the lowest validated concentration (i.e. 0.01 mg kg⁻¹ for LC-MS/MS and 0.05 mg kg⁻¹ for LC-FLD). For LC-FLD, LODs were 0.02 and 0.03 mg kg⁻¹ for glyphosate and AMPA, while LODs were 0.005 mg kg⁻¹ for both glyphosate and AMPA using LC-MS/MS.

The method was applied to 50 rape samples from different areas of Poland. To ensure analysis quality when processing real-world samples, quality control blanks fortified at 0.1 mg kg⁻¹ (RSD 7.3%) were included at five-injection intervals. No glyphosate residues above the limit of detection were found.

4 Conclusion

Simple, fast, efficient, and sensitive methods were developed for glyphosate and aminomethylphosphonic acid determination in rape. Validations demonstrate that the methods are reliable and consistent. Extraction efficiencies and analytical sensitivities are sufficient to address concerns over increasing glyphosate use. The sensitivity and specificity meet the rape glyphosate and aminomethylphosphonic acid in water by online solid-phase extraction-high-performance liquid chromatography-electrospray ionization mass spectrometry, J. Chromatogr. A., 1998, 794, 187-199

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[6] Acknowledgments: This work was supported by the Ministry of Agriculture and Rural Development under the IPP–NRI 2011–2015 Long – Term Program “Protection of cultivated plants with the consideration of food safety, reduction of yield losses and threat to humans, farm animals and the environment” (contract No.HORkor.0660/ IOR2011-2015/2/2013), task No.1.8, and by the Ministry of Science and Higher Education, project ID: SBI-05.

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