

**A Rapid and Sensitive Single Residual Method for Determination of Ethephon in Grape by high performance liquid chromatography-tandem mass spectrometry**

**Um método residual rápido e sensível para a determinação de Ethephon em uvas por cromatografia líquida de alta eficiência e espectrometria de massa em tandem**

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## **ABSTRACT**

This paper describes a rapid (7.0 min) and sensitive (LLOQ 0.1 ng/mL) analytical method for the quantitation of Ethephon in grape. A new method for the detection and quantification of ETP residues in fruit and vegetables was developed. The present study indicates that fruit and vegetables require a rapid and simple cleanup step before using gas chromatograph/mass spectrometry. The recovery and precision of the new method were evaluated by spiking the fruit and vegetable samples with 0.01-0.1 µg/g of ETP. The method is based on High-performance Liquid chromatography-tandem mass spectrometry (LC-MS/MS). The chromatographic separation was achieved on a Hypercarb 2.1x 100 mm 5µm reversed-phase column and a mobile phase containing water/methanol (97:3 v/v, add 1% acetic acid), in isocratic conditions. The target analytes were transferred into a triple quadrupole mass spectrometer equipped with an electrospray ionization source for mass detection. The ion transitions selected for MRM detection were:  $m/z$  142.8 > 106.8,  $m/z$  144.8 > 106.8 and 106.8 > 78.8 for ETP. The linearity of the detector response was demonstrated in the range from 0.01 to 1 µg/g for each analyte with a coefficient of determination ( $R^2$ ) of  $\geq 0.999$ . The method was successfully applied to determination of ethephon in bunch grapes from five free trade fairs in the city of Caruaru, Pernambuco, Brazil.

**Keywords:** Ethephon, LC-MS/MS, Grape, Validation

## **RESUMO**

Este artigo descreve um método analítico rápido (7,0 min) e sensível (LLOQ 0,1 ng / mL) para quantificação de Etefon (ETP) em uvas. Um novo método para a detecção e quantificação de resíduos de ETP em frutas e vegetais foi desenvolvido. O presente estudo indica que frutas e vegetais requerem uma etapa de limpeza rápida e simples antes de usar o cromatógrafo a gás / espectrometria de massa. A recuperação e a precisão do novo método foram avaliadas adicionando-se amostras de frutas e vegetais com 0,01-0,1 µg/g de ETP. O método é baseado em cromatografia líquida de alta eficiência e espectrometria de massa em tandem (LC-MS / MS). A separação cromatográfica foi obtida em uma coluna de fase reversa Hypercarb 2,1x 100 mm 5 µm e uma fase móvel contendo água / metanol (97: 3 v / v, adicionada 1% de ácido acético), em condições isocráticas. Os analitos alvo foram transferidos para um espectrômetro de massa triplo quadrupolo equipado com uma fonte de ionização por electropulverização para detecção de massa. As transições de íons selecionadas para a detecção de MRM foram:  $m / z$  142,8 > 106,8,  $m / z$  144,8 > 106,8 e 106,8 > 78,8 para ETP. A linearidade da resposta do detector foi demonstrada na faixa de 0,01 a 1 µg/g para cada analito com um coeficiente de determinação ( $R^2$ ) de  $\geq 0,999$ . O método foi aplicado com sucesso na determinação do etefon em uvas de cinco feiras livres da cidade de Caruaru, Pernambuco, Brasil.

**Palavras-chave:** Etefon, LC-MS/MS, Uva, Validação

## 1 INTRODUCTION

Ethephon (ETP), 2-Chloroethylphosphonic acid, (Figure 1), is one of most common plant growth regulators (PGRs) and used on various crops to regulate fruit set and flowering for growth and hasten maturity (Abdelgadir, *et al.*, 2010), reduce the risk of lodging (Hau *et al.*, 2000), promote uniform color, loosen fruit, delay flowering reduce plant height (Byers *et al.*, 2003) and increase stalk strength (Basra, *et al.*, 2000). Its mode of action is via liberation of ethylene (its active metabolite) which is absorbed by the plant and interferes in the growth process, including seed germination, fruit maturation and flower wilt (Roberts *et al.*, 1998).

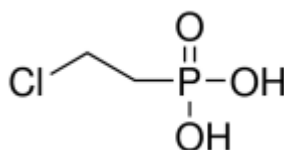


Figure 1. Chemical structure of Ethephon (ETP)

Several HPLC methods for determining ETP levels in human plasma have been described. Previous studies of ethephon used gas chromatography (GC) with a flame photometric detector (FPD) for residue analysis or a nitrogen–phosphorus detector (NPD) (Hurter *et al.*, 1978; Koppen, 1997). However, this approach is rather tedious and time consuming because of the need for derivatization and a cleanup procedure. The strong polarity of ethephon means that it is only weakly retained in reversed-phase liquid chromatography (LC) systems; hence separation by traditional analytical methods is difficult (Xue *et al.*, 2011). In view of the polar and nonvolatile nature of ethephon, LC with tandem mass spectrometry (LC/MS/MS), the ‘workhorse’ in target analysis, should be the most suitable technique for detection and quantitative analysis (Galera *et al.*, 2008; Krauss *et al.*, 2010). The downsides of this procedure are its need for a cleanup procedure and its inapplicability to field incurred samples. Furthermore, the high variability of the recovery (43.6–79.6%) owing to the hydrophilicity and chemical instability of the analyte is another major drawback (Royer *et al.*, 2006). In another study (Takenaka, 2002), ETP residues were detected and quantified in fruit and vegetables using GC/MS after cleanup. It is noteworthy that both of the above mentioned methods require a cleanup procedure prior to analysis.

This paper describes an HPLC-MS/MS method to determine the grape of ETP concentrations. It utilizes precipitation and can analyze over the concentration range of

0.01–1.0  $\mu\text{g}\cdot\text{g}^{-1}$ . The purpose of this study is to evaluate the presence of ethephon in samples of grapes marketed in the Caruaru-PE free trade fairs.

## **2 MATERIALS AND METHODS**

### **2.1 CHEMICALS AND REAGENTS**

Ethephon reference standard was acquired from the United States Pharmacopoeia (Rockville, MD, USA) from the United States Pharmacopea (Rockville, MD, USA). HPLC-grade acetic acid and methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was purified using a MilliQ® system from Millipore (Molsheim, France).

### **2.2 INSTRUMENTATION**

High performance liquid chromatography was done using a chromatograph composed of two pumps (LC 10ADvp), a column oven (CTO 10Avp), an autosampler (SIL 10ADvp), and a system controller (SCL 10Avp), Shimadzu (Kyoto, Japan). The LC equipment was connected to a Quattro Premier XE Mass Spectrometer (Waters, USA). For sample extraction a Jouan M23i refrigerated centrifuge (St. Herblaim, France) was used.

### **2.3 CHROMATOGRAPHY CONDITIONS**

For the LC optimization some analytical columns were evaluated. Reversed phase C18 (Gemini 100 x 3.5 mm, 5  $\mu\text{m}$ ), reversed phase C18 (ACE, 125 x 2.1, 5 $\mu\text{m}$ ) and reversed phase Hypercarb (Thermo, 100 x 2.1 mm, 5 $\mu\text{m}$ ). The mobile phase was achieved by varying the percentage of organic solvent (methanol or acetonitrile) for a short analytical time, the best compromise between separation efficiency, peak shape and stability of the MS signal.

### **2.4 MS/MS conditions**

The LC equipment was operated with negative electrospray ionization (ESI-) interface source. The mass spectrometric parameters (Cone voltage, collision energy, source temperature, desolvation gas, multiplier detection) were optimized to obtain maximum sensitivity at unit resolution. The multiple reaction monitoring (MRM) detection mode was employed to ETP  $m/z$  106.8 > 78.8 (quantification) and 142.8 > 106.8 (confirmation) parent and daughter ion fragments, respectively, with dwell time set at 0.5 s for each transition.

## 2.5 PREPARATION OF WORKING SOLUTIONS AND QUALITY CONTROL STANDARDS

The stock solution of ETP (at  $1.0 \text{ mg.mL}^{-1}$ ) was prepared by dissolving the substance in methanol. The calibration curves for ETP were prepared in grape extract at concentrations of 0.01, 0.05, 0.2, 0.5, 0.8, and  $1.0 \text{ }\mu\text{g.g}^{-1}$ . Quality control (QC) samples were also prepared in human plasma at the following concentrations: 0.02, 0.4 e  $0.8 \text{ }\mu\text{g.g}^{-1}$  (low, medium and high concentration).

## 2.6 SAMPLE PREPARATION

Samples Weigh  $10\text{g} \pm 0.1\text{g}$  of previously crushed fruit and vegetable samples in a centrifuge tube (50 mL). Add 10 mL of the 1% formic acid solution in methanol to the mixture and vortex-mixed for 2 min. The samples were centrifuged at for 6 minute at  $3000 \times g$ . After was transferred 100  $\mu\text{L}$  the supernatant aliquot of the filtered extract to a new vial plus 900  $\mu\text{L}$  of the 1% formic acid solution in water to seal vial, homogenize in Vortex for 5 seconds to glass vials and placed in the autosampler for analysis.

## 2.7 METHOD VALIDATION

Specificity was evaluated by extracting grape samples from a pool of grape. Recoveries of ETP at the three QC concentrations were determined by comparing peak areas of spiked plasma samples with the peak area in solutions prepared with the same nominal concentration. For precision (as relative standard deviation (R.S.D.)) and accuracy (as relative error (R.E.)) studies, samples were prepared at three QC with 6 replicates each, and were analysed in the same day (intra-day precision and accuracy), and analysed in 3 consecutive days (inter-day precision and accuracy). The stability of the solutions and grape extract samples was also evaluated during method validation. ETP stock solutions were analysed at two QC levels (low and high QC, corresponding to 0.02 and  $0.8 \text{ }\mu\text{g.g}^{-1}$ , respectively) both recently prepared or after 48 hours s stored at  $4^\circ\text{C}$ . The stability of ETP was also evaluated in post-extracted samples kept in the autosampler at room temperature ( $23^\circ\text{C}$ ) for 72 or 48 hours, as well as in plasma samples kept at  $-20^\circ\text{C}$  for 15 days and after being submitted to 3 freeze-thawing cycles (24 hours each cycle). All samples described above were compared to freshly prepared ETP samples at the same concentration level.

## 2.8 APPLICATION OF METHOD

The samples used in the experimental development of this work will consist of samples of grapes, mature stage, marketed at the free trade shows of the city of Caruaru-PE, Brazil. Were included samples of the five largest free trade fairs of the city of Caruaru-PE, with satisfactory physical-chemical and organoleptic characteristics.

The selected samples were prepared as follows: the sample was ground to a homogeneous slurry; then remove a portion of approximately 100 g of the homogeneous paste and put it in a disposable aluminum container, close and store it in a freezer at -20 °C.

## 3 RESULTS AND DISCUSSION

### 3.1 LC- MS/MS conditions

Chromatographic separation was achieved using a Hypercarb column (Thermo®) with 100 x 2.1 mm, 5µm. The mobile phase, isocratic conditions, consisted of water/methanol (97:3 v/v), add 1% acetic acid which was filtered, degassed and pumped at a flow rate of 0,25 mL/min. The column oven was set at 20 °C and the injected volume was 9 µL. Retention time for ETP was 3.3 min and analytical run was 7.0 min (Figure 2). The source temperature was optimized at 120 °C, desolvation temperature was 400 °C, and desolvation gas flow was 800 L/h. The capillary voltage was set at 2,8. kV, while optimized cone voltage values for ETP was 16 V. The collision energy was optimized for ETP was 8 V. The multiplier was set at 750V and argon was used as the collision gas at a pressure of  $1.88 \times 10^{-3}$  psi in the collision cell.

### 3.2 METHOD VALIDATION

#### 3.3.1 specificity

The method demonstrated excellent chromatographic sepecificity with no endogenous or metabolite interferences at the retention time for ETP (3.3min). Chromatograms of extracted blank of grape sample containing low (0.01 µg/g) and high (1 µg/g) concentrations, indicated good detector response and baseline resolution for ETP, with an analytical run time of 7 min ( Figure 2). The carry over test shows not interference between samples of an analysis sequence.

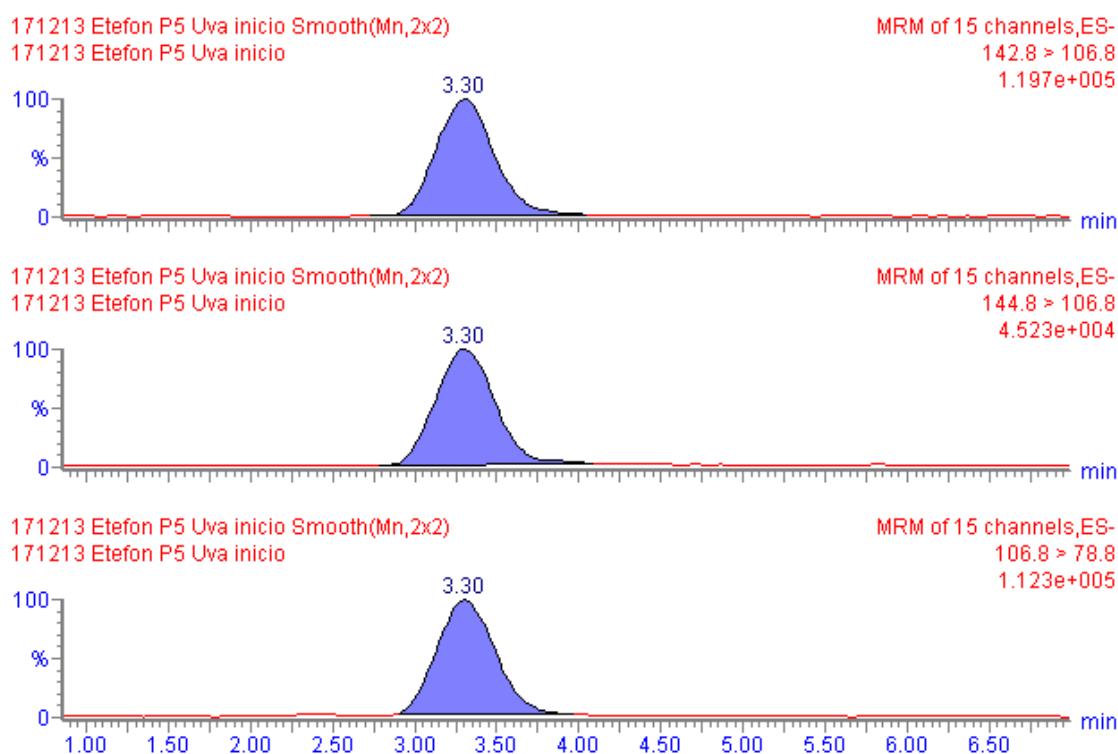


Figure 2. Chromatograms of Etfepon (ETP)

### 3.3.2 linearity, precision, accuracy and recovery

Good linearity was obtained in the concentration range of 0.01 – 1 µg/g (10-1000 ng/g) with a mean correlation coefficient of 0.999 (n= 3 analytical runs, Table 1). The LLOQ was 10 ng/g. The recovery of ETP, using precipitation extraction with 1% formic acid solution in methanol was 98.42%.

Table 1. Accuracy and precision of Linearity.

Spiked concentration (ng.g <sup>-1</sup> )	Determinad concentration (ng.g <sup>-1</sup> ) (Mean ± SD) (n=3)	Precision (%)	Accuracy (%)
10	10.539± 0.238	2.26	105.4
50	50.931± 1.467	2.88	101.9
200	201.126± 5.784	2.88	100.5
500	503.233± 10.078	2.00	100.4
800	805.726 ± 9.762	1.21	100.6
1000	1000.775 ± 1.533	1.53	100,1



Matrix effects were investigated by analysis of spike-after-extraction samples with pure standard solutions at the same concentrations and the results is 97.39% for ethephon. Thus, ion suppression or enhancement from grape matrix was negligible for this method. Intra- and inter-day precision and accuracy results (Table 2) gave satisfactory results in that R.S.D. < 4.51 % and R.E. < 5.63%.

Table 2: Precision and accuracy results of validation

### 3.3.3 Stability studies

Spiked conc. (ng.g <sup>-1</sup> )	Intraday (ng.g <sup>-1</sup> ) (n=6)			Interday (ng.g <sup>-1</sup> ) (n=24)		
	Day 1 Mean (R.S.D.)	Day 2 Mean (R.S.D.)	Day 3 Mean (R.S.D.)	Mean	Prec.	Acc. (R.E.)
10	10.27 (2,1%)	10.77 (1,8%)	10.59 (4,9%)	10.56	4.51	105.63
20	20.55 (2,4%)	20.55 (3,8%)	20.49 (1,5%)	20.53	3.23	102.36
400	398.48 (1,9%)	397.97 (1,6%)	396.23 (0,8%)	397.51	1.05	99.58
800	800.77(5,8 %)	800.27 (3,1%)	800.76 (3,4%)	800.93	4.03	100.01

The stability data of ETP in grape sample under different temperature, time conditions and freeze-thaw was demonstrated as the calculated concentrations for the controls did not significantly decrease over the course of the study (Table 3).

Table 3. Stability data of assay of Ethephon

(A) Stability Test	Ethephon (n=5) Conc. ng/g	Precision (R.S.D.) (%)	Accuracy (R.E.) (%)
Bench top stability	20	5.32	-1.26
	800	3.77	-1.32
Autosampler stability	20	1.26	-1.22
	800	1.91	-0.89
Freeze-thaw stability	20	3.80	-2.44
	800	1.21	-1.45
15-days storage stability	20	3.59	-4.71
	800	4.58	-5.40
Solution stability	20	5.22	-1.23
	800	3.44	0.97



**3.3.4 Study of grape samples**

The developed method was successfully applied to quantification of the level of ethephon in the study of grape samples. Ethephon analysis is mandatory for several foodstuffs especially table grapes in the coordinated community control program laid down in Commission Regulation. The tolerance level of ethephon in Brazil for grapes is 0,1 µg/g, all the samples, coming from the five free fairs of the city of Caruaru-PE, evaluated presented values under the residual maximum limit. In this way, the tested products present conforming to the consumption, once the level of ETP is within the tolerated limits for this type of culture (grape). The concentrations found in the samples are in Table 4.

Table 4: Assay of Ethephon

Sample code	Determinad concentration (ug.g <sup>-1</sup> ) (Mean ± SD) (n=3)
AM_CARUARU_01	0,02 ± 0.0123
AM_CARUARU_02	0,06 ± 0.0145
AM_CARUARU_03	0,01 ± 0.0078
AM_CARUARU_04	0,03 ± 0.0134
AM_CARUARU_05	0,01 ± 0.0045

**4 CONCLUSION**

We have described a simple, rapid and sensitive LC-MS/MS method for the quantitation of ETP in grape samples, which showed acceptable precision and adequate sensitivity. The major advantages of this method are the simple sample preparation, the short run time (7 min) for high throughput analysis and good sensibility, which are all important characteristics when dealing with large batches of samples. This method has been successfully applied to determination of ETP grape samples. And all the samples evaluated from this work presented values within the limits established by Anvisa.

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