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Determination of Ethoxyquin in Paprika by High-Performance Liquid Chromatography

P. Viñas, M. Hernández Córdoba* & C. Sánchez-Pedreño

Department of Analytical Chemistry, Faculty of Chemistry,
University of Murcia, 30071 Murcia, Spain

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ABSTRACT

*Ethoxyquin is an antioxidant agent used to retard the decomposition of some pigments. A new method for the determination of ethoxyquin in paprika avoiding previous separation steps from other coloured substances is proposed. The analysis is carried out by reverse-phase high-performance liquid chromatography using the gradient elution technique and UV detection at 270 nm. Using fluorimetric detection with excitation at 311 nm and emission at 444 nm, a detection limit of 0·2 μg/ml can be reached. The method is applied to the determination of ethoxyquin in commercial samples of paprika (*Capsicum annum*).*

INTRODUCTION

Since paprika is a natural, non-toxic substance with a marked pigmenting potency, it has been widely used in food and pharmaceutical industries, cosmetics, etc. The typical red colour of paprika is due to carotenoids. These compounds are altered by oxygen and oxidative reactions, light and heat (De la Torre & Farré Rovira, 1975). During processing and storage, paprika powder is frequently exposed to these deleterious factors. Ethoxyquin (EQ) has been proved to be a useful agent for the protection of the natural red colour of paprika.

* To whom correspondence should be addressed.

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EQ (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is an antioxidant agent which has been used to retard decomposition of some pigments, to prevent the formation of brown spots on apples and as a preservative in foods. The tolerated levels for this antioxidant vary in different countries. Some studies concerning the determination of EQ in apples (Winell, 1976; Ernst & Verveld-Röder, 1979; Olek *et al.*, 1983), milk (Perfetti *et al.*, 1983), waste-waters (Victor *et al.*, 1984), eggs and chick tissue (Van Deren & Jaworski, 1967, 1968) and spices (Perfetti *et al.*, 1981; Fujinuma *et al.*, 1982; Uchiyama & Uchiyama, 1983) have been reported. However, the proposed methods are not simple. Most of them require liquid–liquid solvent-extraction steps in order to separate EQ from other compounds prior to the chromatographic determination and these separation processes are time consuming.

In the present study, a new procedure for the determination of EQ in paprika samples is reported. Reverse-phase high-performance liquid chromatography (HPLC) with both spectrophotometric (270 nm) and fluorimetric detection (excitation 311 nm and emission 444 nm) applying the gradient elution technique is used. The chromatographic system allows the determination of EQ in the presence of the paprika carotenoids avoiding the previous separation step.

MATERIALS AND METHODS

Apparatus

The HPLC system consisted of a Perkin–Elmer Series 4 liquid chromatograph operating at room temperature and with a flow-rate of 2 ml/min; a Perkin–Elmer LC-85B variable wavelength scanning spectrophotometric detector with a 8-μl flow-cell and wavelength set at 270 nm; a Perkin–Elmer 3000 variable wavelength fluorimeter with a flow-cell model 5212 using 311 nm for excitation and 444 nm for emission; a 7125-075 Rheodyne injector valve with a 6-μl sample loop and a Perkin–Elmer Sigma 15 chromatography data station. The column was 12·5 × 0·46 cm i.d. stainless steel packed with Spherisorb ODS-2 with a particle size of 5 μm.

Reagents

Solvents

Acetonitrile and ethyl acetate (Romil Chemicals, Loughborough, Leicester, UK) were liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The solvents were degassed by sparging them with helium gas.

EQ

EQ was obtained from Sigma (St Louis, MO, USA) and no other substances were detected by mass spectrometry. The solution was prepared from the commercial product, without further purification, by dissolving 50 mg in 50 ml of ethyl acetate. The solution was prepared daily and stored in a dark bottle in the cold. Working solutions were obtained by dilution with ethyl acetate immediately before the measurements.

Calibration graph for EQ

Standard solutions of EQ in the concentration range 2–50 μg/ml were prepared by appropriate dilutions with ethyl acetate from the stock solution. These solutions were kept in the dark to avoid decomposition of EQ by light. The chromatographic conditions were as follows: mobile phase, acetonitrile–water 80:20; flow-rate, 2 ml/min; room temperature; sample-loop, 6-μl; detection wavelength, 270 nm. When fluorimetric detection was required, chromatographic conditions were as previously described, setting the excitation and emission wavelengths at 311 and 444 nm, respectively. Under these conditions, a linear calibration graph from 0·2–4 μg/ml was obtained. Calibration graphs were prepared by plotting concentration against peak area.

Determination of EQ in paprika

A paprika sample of 2 g was accurately weighed and extracted with 20-ml of ethyl acetate in a closed beaker. The solution was stirred for 10 min, filtered and diluted to 25 ml with ethyl acetate in a volumetric flask. Again, these solutions were kept in the dark when not in use. The samples were injected into the chromatograph and a gradient elution technique was used: the initial chromatographic conditions were acetonitrile–water (80:20) flowing during 1·5 min; the linear gradient elution started from this mobile phase to 100% acetonitrile in 1 min. This latter mobile phase was held for 3 min. Then, a second linear gradient was carried out in ethyl acetate–acetonitrile (95:5) for 1 min. Isocratic elution was then held for 3 min and, finally, initial conditions were reestablished in 10 min. The flow-rate was 2 ml/min throughout the gradient. Other chromatographic conditions were the same as those in the calibration graph.

RESULTS AND DISCUSSION

The organic extracts of paprika samples contained many other coloured substances which can overlap with EQ as well as being strongly retained in

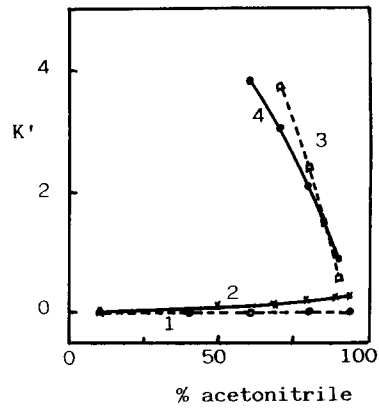

Fig. 1. Dependence of the capacity factor of EQ on the composition of the mobile phase. (1) acetonitrile–ethyl acetate; (2) acetonitrile–ethyl acetate–1% ammonium acetate; (3) acetonitrile–water; (4) acetonitrile–ammonium acetate. Flow-rate, 2 ml/min; UV detection, 270 nm.

the column decreasing its performance. In a previous report (Perfetti *et al.*, 1981), EQ was extracted from the paprika with hexane and partitioned into HCl. The solution was adjusted to pH 13–14 and EQ was re-extracted into hexane. The hexane layer was then evaporated to dryness and dissolved in acetonitrile prior to the injection into the chromatograph. A similar procedure involving iso-octane as the extractant has also been proposed (Fujinuma *et al.*, 1982). However, it is apparent that for routine analysis, these processes are time consuming. The aim of our work was to develop a simpler and faster method useful for routine purposes.

The chromatographic behaviour of EQ with a set of solvents typically used for HPLC was studied at a fixed wavelength of 270 nm. Figure 1 shows the dependence of capacity factors on mobile phase composition. The capacity factor (K') was calculated in the standard fashion $K' = (t_R - t_M)/t_M$, where t_R is the retention time for the component and t_M is the mobile phase void volume. EQ was not retained in the column with the use of the mobile phase acetonitrile–ethyl acetate and eluted very close to the void volume (see curve 1). On the other hand, K' of EQ only showed a slight increase after the addition of 1% ammonium acetate to the acetonitrile (curve 2). These results indicated that ethyl acetate was not an adequate solvent since EQ eluted at the retention time for a non-retained peak. Therefore, acetonitrile–water mixtures were tried (curve 3). The K' values of EQ were then significantly dependent on the mobile phase composition; at the higher water concentration, EQ was more strongly retained and was not eluted as a sharp peak. Similar results were found with acetonitrile–ammonium acetate mixtures (curve 4). On the basis of these results, the optimal difference in retention times between EQ and non-retained compounds was achieved when a 80:20 acetonitrile–water mixture was used as a mobile phase.

Figure 2 shows some typical chromatograms for standard EQ at different concentrations using spectrophotometric detection. Calibration graphs

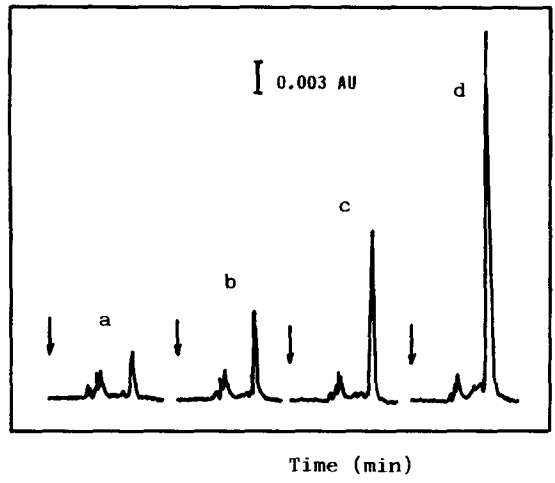

Fig. 2. Calibration graph for EQ standard. Mobile phase, acetonitrile–water 80:20; flow-rate, 2 ml/min; sample loop, 6-μl; spectrophotometric detection at 270 nm. EQ, μg/ml: (a) 5·08; (b) 10·15; (c) 20·30; (d) 42·30. Arrows indicate the injection.

were obtained by plotting the peak area against the antioxidant concentration and were linear in the range 2–50 μg/ml (12–300 ng). The relative standard deviation (RSD) values for 20 and 40 μg/ml of EQ (10 determinations at each level) were 2·4 and 1·9%, respectively. When fluorimetric detection was used, linear calibration graphs in the 0·2–4 μg/ml (1·2–24 ng) EQ concentration range were obtained. The RSD for 0·5 μg/ml of EQ (10 measurements) was 3·2%.

Several solvents were tried to prove that EQ was quantitatively extracted from the paprika. Good results were obtained with ethyl acetate and acetonitrile. The latter was discarded owing to its toxicity and ethyl acetate was used.

The next step was the selection of an adequate mobile phase allowing the simultaneous separation and determination of EQ in the presence of the carotenoid pigments avoiding the previous isolation of the antioxidant. Figure 3 depicts the elution profiles corresponding to the injection of (1) a paprika sample and (2) EQ standard with different mobile phases. The chromatograms indicated that the K' values of EQ and carotenoids were significantly dependent on the mobile phase composition. Thus, in an acetonitrile–water 80:20 mixture (see chromatograms A), part of the injected coloured material was weakly retained and produced a broad peak at a short retention time, while the rest of the pigments were all strongly retained in the column. Similar results were obtained when 100% acetonitrile was used as a mobile phase (chromatograms B). The addition of ethyl acetate to the mobile phase allowed the elution of the more strongly retained carotenoids, decreasing the capacity factors of the pigments

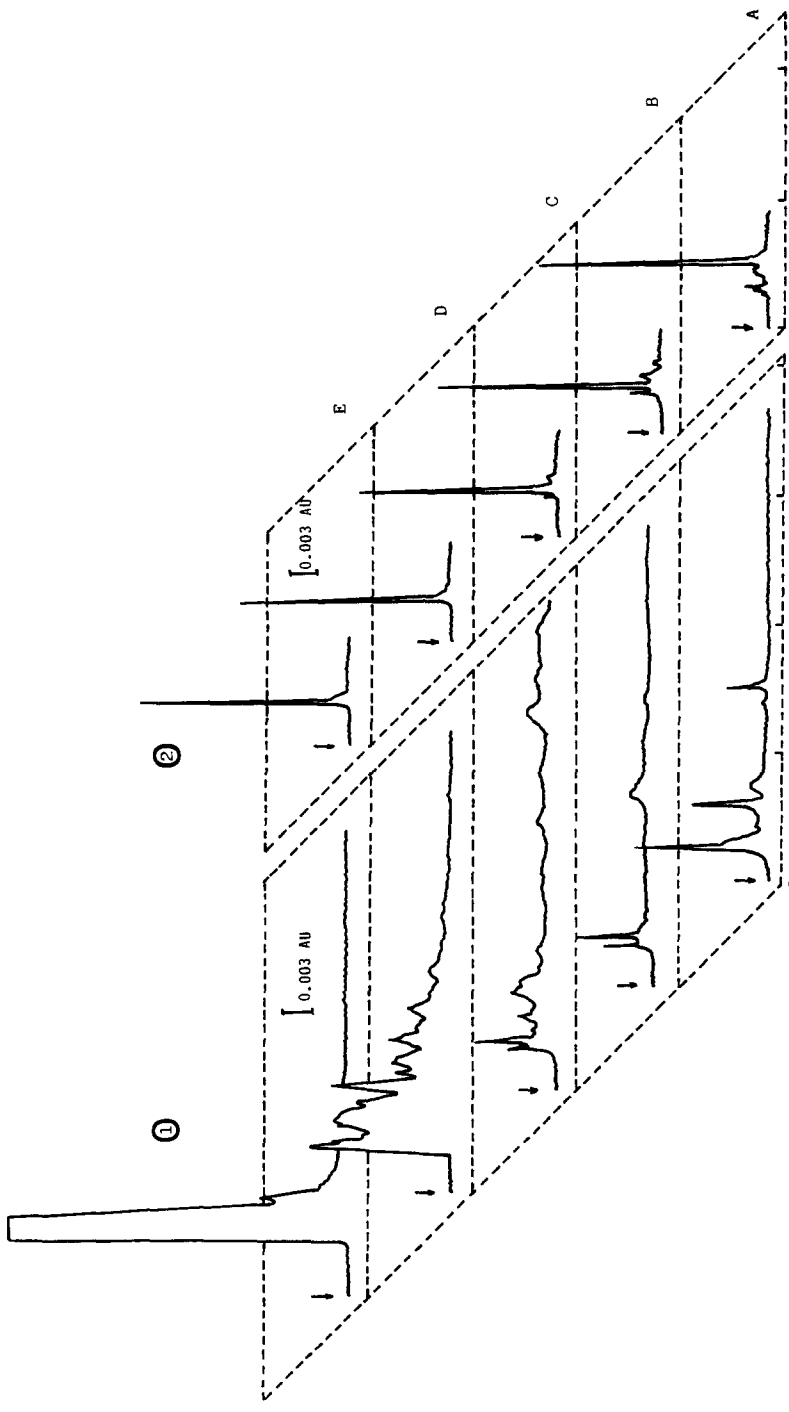

Fig. 3. Elution profiles of (1) a paprika sample, and (2) EQ (30 μg/ml) with different mobile phases. Flow-rate, 2 ml/min; sample-loop, 6-μl; spectrophotometric detection at 270 nm. Mobile phase, %: (A) acetonitrile–water 80:20; (B) acetonitrile 100; (C) acetonitrile–ethyl acetate 80:20; (D) acetonitrile–ethyl acetate 50:50; (E) acetonitrile–ethyl acetate 20:80.

when the proportion of ethyl acetate increased (chromatograms C, D and E). However, the acetonitrile–ethyl acetate mixture was not a good solvent since EQ and carotenoids eluted together and the separation was not possible.

A linear solvent strength gradient elution was then used to optimize the resolution of the mixture. Gradient elution allowed a significant reduction of the total analysis time and a separation of the multiple components having a wide range in polarity. Thus, two ternary elution gradient solvent systems at a flow-rate of 2 ml/min were tried. In all cases, an interval of at least 10 min was allowed for re-equilibration before each run. Table 1 shows the elution gradient programmes which produced the chromatograms shown in Fig. 4.

With 100% acetonitrile (programme B) as the starting eluent, the gradient was both easier and quicker to perform; however, EQ eluted very close to the initial non-retained compounds. The gradient A, starting with the 80:20 acetonitrile–water mixture, is longer than gradient B, but it allowed a better resolution between EQ and non-retained pigments since EQ eluted later. In both instances, the more strongly retained pigments were eluted together at the end of the gradient run. The results suggest that the optimal difference in retention times was achieved with the acetonitrile–water mixture as the starting eluent (gradient A). Thus, this was the gradient programme selected for the rest of the experiences. Similar chromatograms were obtained when fluorimetric detection was used.

The mobile phase flow-rate was not a significant factor affecting the separation of EQ and other constituents in the range studied, 0·5–2·0 ml/min. Hence, a 2 ml/min flow-rate providing a quicker elution of

TABLE 1
Elution Gradient Programmes for the Separation of EQ and Carotenoids

System	*Stage*	*Time (min)*	*Mode*	*Elution solvent (%)*		
				MeCN	*Water*	*Ethyl acetate*
A	Equil	10	Step	80	20	0
	Start	1·5	Step	80	20	0
		1	Linear	100	0	0
		3	Step	100	0	0
		1	Linear	5	0	95
	End	3	Step	5	0	95
B	Equil	10	Step	100	0	0
	Start	2	Step	100	0	0
		1	Linear	5	0	95
	End	3	Step	5	0	95

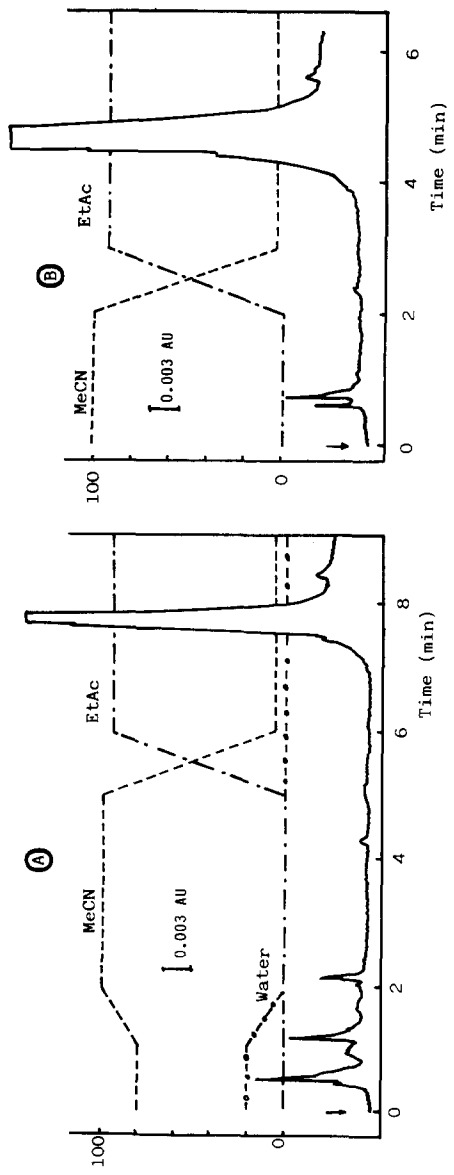

Fig. 4. Elution profiles of a paprika sample with different gradient elution programmes. Flow-rate, 2 ml/min; sample loop, 6-μl; detection, 270 nm. (A) gradient A; (B) gradient **B** (see Table 1).

TABLE 2
EQ Found in Commercial Paprika Samples

Sample	*Ethoxyquin found (μg/g)*	
	No EQ added	*125 μg EQ added*
1	158	286
2	—	126
3	165	294
4	132	254
5	158	288
6	182	310
7	100	228
8	235	364
9	205	337
10	—	127
11	61	184
12	34	159
13	29	156

the carotenoids at the end of the gradient was used throughout this investigation.

After concluding the optimization scheme for the separation of EQ and the paprika pigments, several paprika samples containing EQ at different concentration levels were analysed. First of all, the optimal procedure for the extraction of paprika samples was tested. Results showed that EQ is completely extracted after approximately 5 min of stirring with ethyl acetate. Therefore, an extraction time of 10 min was selected.

Results for the EQ content in sweet paprika samples using the spectrophotometric detection procedure are presented in Table 2. Six aliquots were taken for each sample and each one of the extracts was three times injected into the chromatograph. Paprika samples were also analysed by adding 125 μg of EQ standard to the solution. Results are in concordance with those obtained for non-spiked samples.

The recovery of EQ was determined by analysing paprika samples spiked with EQ at levels of 10–30 μg/ml; the results are shown in Table 3.

Experiments were carried out to study the reproducibility of the method. In order to determine the instrument stability, ten injections of the same paprika extract were carried out using spectrophotometric detection and a relative standard deviation of 3·1% was obtained. To calculate the precision of the whole procedure, ten separate extractions were performed from the same sample and the solutions were chromatographed. The RSD was now 4·2%.

TABLE 3
Recovery of EQ from Paprika following the Standard Addition Method

Sample	*EQ* (μg/g)	*EQ taken* (μg/ml)	*EQ added* (μg/ml)	*Total EQ found* (μg/ml)	*Recovery* (%)
Paprika 11	61	4·9	—	4·9	—
(2·004 3 g)			9·6	14·4	99·3
			19·2	25·3	104·9
			28·7	34·6	103·0
Paprika 4	132	10·6	—	10·6	—
(2·000 1 g)			9·6	19·5	96·5
			19·2	30·8	103·3
			28·7	41·0	104·3
Paprika 6	182	14·6	—	14·6	—
(2·001 1 g)			9·6	25·2	104·1
			19·2	35·0	103·5
			28·7	45·7	105·5

CONCLUSION

A new procedure for the determination of EQ in paprika by HPLC is presented. Using a gradient elution technique, EQ is determined in the presence of paprika carotenoids without previous separation steps. In order to improve the sensitivity, detection can be carried out by the fluorimetric technique. The procedure can be applied to the routine determination and quality control analysis of EQ in commercial paprika samples.

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