Journal of Chromatography, 262 (1983) 340–345
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,786

Note

Thin-layer chromatography–fluorometry of ethoxyquin using Triton X-100

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(First received August 4th, 1981; revised manuscript received February 15th, 1983)

Ethoxyquin (EQ; 6-ethoxy-1,2,-dihydro-2,2,4-trimethylquinoline) is used as an antioxidant for spices, feeds and other foods. There have been several reports[1–7] concerning determinations of EQ but the methods involved were not simple. A simple analysis of EQ was therefore established by use of a fluorescence-enhancement method[8] on a thin-layer plate, and used for the determination of EQ in spices.

This paper deals with the selection of the most suitable selective spraying reagent for EQ, the improvement of the spraying method so as to achieve uniform fluorescence-enhancement of EQ when using a conventional spraying method employing a rubber bulb, the thin-layer chromatographic (TLC) conditions and the extraction of EQ from spices containing many other coloured substances. The interference of aflatoxins with EQ is also discussed.

EXPERIMENTAL

Materials and apparatus

Most of solvents were of reagent grade and were distilled before use; however Triton X-100, liquid paraffin and glycerol (Wako, Osaka, Japan) were used as supplied. Reagent-grade EQ was purchased from Tokyo Chemical Industry, Tokyo, Japan.

The fluorescence spectra and the relative fluorescence quantum number (RFQN) were recorded with a Hitachi Model MPF-3A spectrofluorimeter. The RFQN of the total fluorescence was taken as the ratio (area shown for a test solution)/(area shown for a hexane solution) as described previously[8]. Additionally, the RFQN of EQ in *n*-hexane solution was assigned a value of 1.0 with an excitation wavelength of 360 nm.

Extraction of EQ from spices

Spices (5 g) were cut into fine pieces. If they contained an aqueous component, they were dried by mixing with anhydrous sodium sulphate. The spices were extracted with 80 ml of *n*-hexane and the extract was filtered through a filter paper. The residue was again extracted with 20 ml of hexane in the same manner. The combined extract was subsequently shaken with two 50-ml portions of 0.4 *M* sulphuric acid and containing 2% sodium sulphate for 1 min. The aqueous phase was made alkaline by

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addition of 12 ml of 6 *M* sodium hydroxide and extracted with one 50-ml and one 40-ml portion of *n*-hexane by shaking for 1 min. The combined *n*-hexane extract was washed with a small quantity of water, dried over anhydrous sodium sulphate and concentrated to 0.5 ml.

TLC–fluorodensitometry

A 10-μl portion of the prepared sample solution was spotted on to a silica gel plate without fluorescent indicator (E. Merck, Darmstadt, G.F.R.). At the same time, the standard EQ solutions were spotted on to the plate. The spots were then developed with benzene or methanol containing 500 ppm of 2,6-*tert.*-butyl-*p*-cresol (BHT). The developed plate was allowed to stand for 1 h in the dark to allow the developing solvent to evaporate homogeneously from the plate under ambient conditions.

The developed plate was sprayed uniformly at the position of the EQ spot with a mixture of Triton X-100 and benzene (1:2) using a conventional hand-worked rubber-bulb sprayer, until the surface of the thin layer became semitransparent. Immediately after spraying, the benzene component of the reagent was evaporated by blowing cool air using an electric hair dryer in order to fix only the Triton X-100 on the thin layer.

Qualitative analysis of EQ was carried out by observation of its white blue-fluorescent spot on a thin-layer plate under ultra-violet light at 365 nm. Quantitative analysis was conducted by fluorescence measurements on a TLC–fluorodensitometer. Spots were excited at 365 nm and the fluorescence was measured with a 430-nm interference filter. The sample and standard EQ spots were simultaneously scanned in the parallel direction to the original line. The fluorescence intensities of EQ were counted as relative photon numbers recorded with a integrator.

RESULTS AND DISCUSSION

Choice of spray reagent

The fluorescence spectra of EQ in most organic solvents have a maximum excitation wavelength around 360 nm and a maximum emission wavelength around 430 nm. However, EQ itself lost its fluorescence on a thin layer when the solvent was volatilized.

In order to maintain the fluorescence of the EQ spot on the thin layer and, indeed, to enhance it, the fluorescence-enhancement method, *i.e.* spraying with non-volatile and viscous solvents, was investigated. We had previously reported[8,9] that the two main factors involved in this method were the fluorescence productivity of the reagent for a fluorescent compound and the transmigration of the compound into the reagent.

When the fluorescence productivity was expressed as a RFQN, the RFQNs of EQ in various solvents could be seen to decrease linearly with increasing dielectric constant of the solvent, except for pyridine, acetic acid, liquid paraffin and Triton X-100, as shown in Fig. 1. This indicated that the fluorescence productivity was high in a solvent of low dielectric constant. On the other hand, it was presumed that the high RFQN values of liquid paraffin and Triton X-100 may depend upon the high viscosity of these solvents, as previously reported[9] and the low values of acid or alkaline solvents such as pyridine and acetic acid may be due to a change in the chemical conformation.

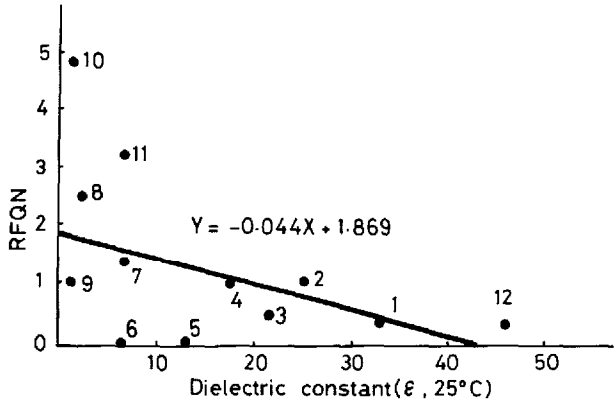


Fig. 1. Relationship between dielectric constant of solvent and relative fluorescence quantum number (RFQN) of EQ (2.8 μg/ml) dissolved in the solvent. The line was drawn from the data of the low viscous solvents except for points 5, 6, 10, 11 and 12. 1 = Methanol; 2 = ethanol; 3 = acetone; 4 = butanol; 5 = pyridine; 6 = acetic acid; 7 = ethyl acetate; 8 = benzene; 9 = *n*-hexane; 10 = liquid paraffin; 11 = Triton X-100; 12 = glycerol.

When the transmigration of the EQ on the thin layer into the solvent phase was substituted for the TLC R_F value using the corresponding solvents as a developing solvent, the R_F values are seen to increase with rising dielectric constant up to ε 6.0, as shown in Fig. 2. Triton X-100 showed a maximum value but liquid paraffin had very small values compared to other viscous solvents; *i.e.* liquid paraffin and Triton X-100 both had high viscosities and fluorescence productivities but liquid paraffin showed a much lower R_F value than Triton X-100. Triton X-100 was therefore expected to be an excellent spray reagent on the basis that the fluorescence enhancement on a thin-layer plate was proportional to the product of the RFQN and the R_F as described previously[9].

Various spraying reagents containing Triton X-100 were examined for EQ spots on a silica gel plate as shown in Table I. A high fluorescence enhancement was

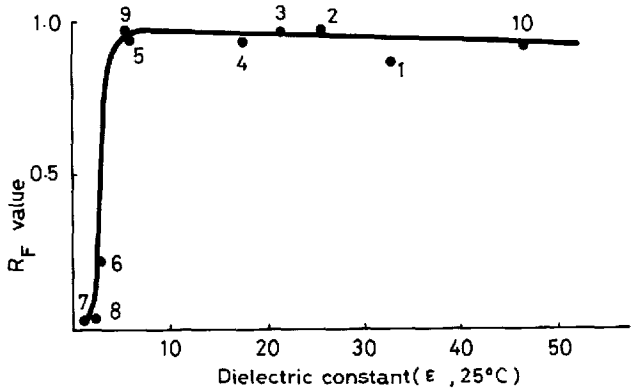


Fig. 2. Relationship between dielectric constant of solvent and the R_F value of EQ (56 ng per spot) on a silica gel plate using the corresponding solvent as a developing solvent. 1 = Methanol; 2 = ethanol; 3 = acetone; 4 = butanol; 5 = ethyl acetate; 6 = benzene; 7 = *n*-hexane; 8 = liquid paraffin; 9 = Triton X-100.

TABLE I

FLUORESCENCE ENHANCEMENT OF EQ PRODUCED BY SPRAYING WITH VARIOUS REAGENTS

Spots (56 ng) of EQ were spotted on to a silica gel plate. The low viscosity solvents in the above systems were added to facilitate easy and smooth spraying.

Reagent system	*Relative fluorescence intensity**
Not sprayed	1
Triton X-100–chloroform (1:2)	218
Triton X-100–benzene (1:2)	220
Triton X-100–liquid paraffin–benzene (1:1:2)	120
Triton X-100–liquid paraffin–benzene (0.2:1:2)	10
Liquid paraffin–benzene (1:2)	6
Glycerol–methanol (1:2)	22

* The fluorescence intensity of unsprayed EQ was taken as 1.

obtained for the Triton X-100 systems containing chloroform or benzene (greater than 200 times), compared to a small one for the liquid paraffin and glycerol systems.

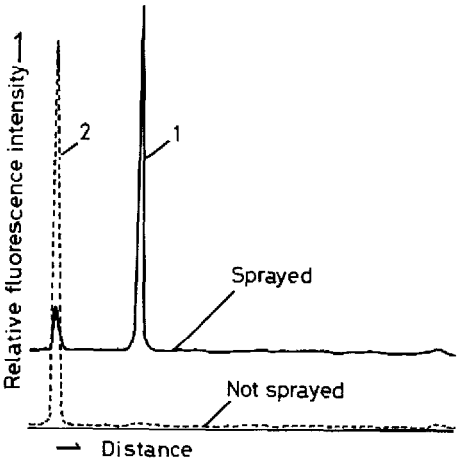
EQ on a thin-layer plate was detected as a white blue-fluorescent spot by spraying a mixture of Triton X-100 and benzene or chloroform. The fluorescence spectra had their maximum excitation wavelength at 434 nm, similar to that of EQ dissolved in Triton X-100. A mixture of Triton X-100 and benzene gave a slightly higher intensity than a mixture of Triton X-100 and chloroform, and also made the fluorescence stable for more than 15 h. As chloroform caused the fluorescence intensity of EQ to fall, it was necessary to evaporate completely the remaining chloroform from the plate. The most suitable spray reagent was therefore eventually determined to be the 1:2 mixture of Triton X-100 and benzene. Additionally, a mixture of triethanolamine and isopropanol (2:8)[13], which has often been used for fluorescence enhancement of Dns derivatives, was not so effective in EQ fluorescence enhancement compared with the Triton X-100 mixture.

Improvement of spraying method

It had previously been found difficult to spray reagents uniformly by a conventional rubber-bulb method to enhance fluorescence intensity at a constant rate[10]. The rubber-bulb spraying method was therefore improved. The thin layer containing EQ was sprayed with the reagent in a line parallel to the origin until the surface of the thin layer became sufficiently semi-transparent so as to be able to yield a sufficient quantity of Triton X-100. Immediately after spraying, cool air was blown on to the sprayed zone using an electric hair dryer in order to evaporate any volatile components. On subsequent fluorodensitometry, the fluorescence intensities of the EQ spots were consistently found to be enhanced more than 200 times. The coefficient of variation (C.V.) for the fluorescence-enhanced intensities of EQ was 2.6%.

TLC of EQ in spices

The *n*-hexane extract of EQ from spices contained many coloured components which overlapped with the EQ spot on the thin-layer plate. These colours were suc-



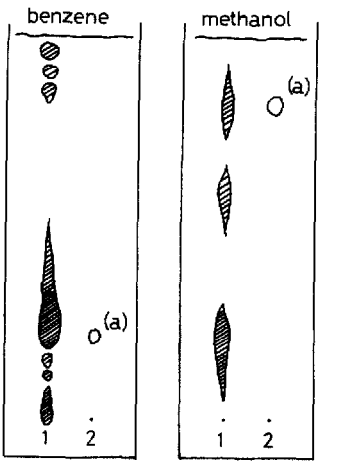


Fig. 3. Thin-layer chromatograms of the initial *n*-hexane extract from EQ-added paprika (1) and the extract after acid–alkaline treatment (2) on a silica gel plate using benzene or methanol. (a) = fluorescent spot of EQ under UV light at 365 nm.

Fig. 4. Fluorodensitometric scanning (reflectance mode) of 50 ng per spot of EQ (1) and 5 ng per spot of aflatoxin B_1 (2) developed on a silica gel plate in benzene, followed by spraying with the reagent Triton X-100–benzene (1:2).

cessfully eliminated by acid and alkaline treatment. Fig. 3 shows the thin-layer chromatogram of an *n*-hexane extract of an EQ-added spice sample.

Although EQ itself is readily oxidized and decomposed in air, it was stable when an *n*-hexane solution was evaporated at 40°C using a rotary evaporator. In addition, a 95%-pure sample of EQ was kept for 10 min even under dry conditions. It was, therefore, possible to adjust the final volume by the addition of 0.5 ml of *n*-hexane to the residue immediately after the sample had been completely dried out at 40°C.

When EQ was developed on a silica gel plate in the benzene or methanol solvent systems, the R_F values were 0.22 and 0.82, respectively. Some 2,6-*tert*.-butyl-*p*-cresol (BHT, 500 ppm) was added to the developing solvents to avoid decomposition of EQ. The BHT always covered the EQ spot in both solvent systems, made the EQ fluorescence stable and also played a role in the greater fluorescence enhancement of EQ.

Determination of EQ

When the developed and sprayed plate was exposed to ultraviolet light at 365 nm in the dark, 40 ng of EQ was detected by the naked eyes as a white blue-fluorescent spot in spite of the scanty fluorescence of EQ itself on thin layer plates. The detection limit of EQ in spices was 0.4 ppm without disturbance by colours derived from spices. On the other hand, when the fluorescence intensity of EQ was measured with a fluorodensitometer, a linear relationship between fluorescence intensity and amount of EQ was found in the range 30–120 ng per spot, *i.e.*, the range over which EQ could be determined in spices was 0.3–1.2 ppm.

When 1.0 ppm of EQ was added to red pepper and paprika, the recoveries were 80.1 (n = 3, SD = 1.1) and 85% (n = 3, S.D. = 1.0), respectively. By means

of this method, red pepper, paprika, white pepper, black pepper and chilli pepper were bound to be not contaminated with EQ but a 0.6 ppm concentration of EQ was detected in chilli pepper obtained on the Japanese domestic market.

This qualitative method therefore makes it possible to examine EQ quickly and simply as a screening test. If a fluorodensitometer is available, quantitative analysis of EQ on a thin-layer plate is possible.

Disturbance of EQ determination by aflatoxin B_1

It had been reported that the prescence of aflatoxin B_1 (micotoxin) in the sample interrupted EQ determination[11,12]. However, in this method, aflatoxin B_1 did not interfere with the EQ spot in the thin-layer chromatogram developed with benzene as shown in Fig. 4. Spraying with Triton X-100 mixture, furthermore, enhanced the fluorescence intensity of EQ more than 200 times, but lowered the fluorescence intensity of aflatoxin B_1 by one eighth. The other aflatoxin also gave no interference with EQ determination.

REFERENCES

1 H. R. Weilenmann, J. Hurter, K. Stoll and A. Temperli, *Lebensm. Wiss. Technol.*, 5 (1972) 106.
2 J. M. VanDeren, Jr. and E. G. Jaworski, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 712.
3 J. M. VanDeren, Jr. and E. G. Jaworski, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 844.
4 J. M. VanDeren, Jr. and E. G. Jaworski, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 537.
5 S. C. Witt, E. M. Bickoff and G. O. Kohler, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 167.
6 H. K. Dahle and J. U. Skaare, *J. Agr. Food Chem.*, 23 (1975) 1093.
7 K. Fujinuma, M. Kanmuri, K. Nakazawa, M. Nakasato, T. Ariga and T. Naoi, *Abstracts of papers, 39th meeting of Food Hygienic Society of Japan*, 1980, p. 39.
8 S. Uchiyama and M. Uchiyama, *J. Chromatogr.*, 153 (1978) 135.
9 S. Uchiyama and M. Uchiyama, *J. Liquid Chromatogr.*, 3 (1980) 681.
10 S. Uchiyama, T. Kondo and M. Uchiyama, *Bunseki Kagaku (Jap. Anal.)*, 26 (1977) 762.
11 H. J. Issaq, E. W. Barr and W. L. Zielinski, Jr., *J. Chromatogr.*, 132 (1977) 115.
12 R. T. Gallagher and H. M. Stahr, *J. Agr. Food Chem.*, 28 (1980) 133.
13 N. Seiler and M. Wiechman, *Z. Anal. Chem.*, 220 (1966) 109.