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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DENATONIUM BENZOATE IN RAPESEED OIL

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SUMMARY

A method for the determination of denatonium benzoate [benzyldiethyl(2,6-xylylcarbamoylmethyl)ammonium benzoate] in rapeseed oil has been developed which utilizes a simple extraction and concentration technique followed by reversed-phase high-performance liquid chromatography. The column eluent was monitored at 210 nm and peak area data was generated by a computing integrator. Detection was possible below 5 ng and calibration curves were linear to 100 ng or more.

INTRODUCTION

The rate of duty assessed on rapeseed oil imported into the U.S.A. varies considerably depending upon its intended use and whether or not it has been rendered unfit for use as a food. In rendering vegetable oils, such as rapeseed oil, unfit for use as food, a denaturant is commonly added. One such substance, specified in government regulations as acceptable for use in the denaturing of vegetable oils, is denatonium benzoate at a level of not less than 2 ppm.

A number of methods have been proposed for the quantitation of denatonium benzoate. The older of these generally involve colorimetric reactions[1,2] or thin-layer chromatography[3], methods which suffer from a lack of specificity or quantitative accuracy and may be extremely time-consuming. A more recent method has appeared which utilizes high-performance liquid chromatography (HPLC) to determine denatonium benzoate in alcoholic toilet preparations[4]. However, in this method, which utilizes a silica gel column, denatonium benzoate is not completely resolved from the solvent "front" and appears simply as a shoulder on a tailing edge. To improve the chromatography, it was decided to investigate the use of chemically bonded phases. Good results have been achieved with these phases in the separation of other quaternary ammonium salts[5,6]. The HPLC procedure selected is reversed-phase consisting of separation on a chemically bonded cyano-type polar phase on microparticulate silica with a mobile phase of acetonitrile–water (60:40) containing 0.01 *M* sodium chloride. The presence of an ionic specie in the mobile phase proved essential and the system afforded baseline separation of denatonium benzoate from the solvent "front". The separation of denatoniu n benzoate from a viscous, oily matrix presented

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more formidable problems than those encountered by others[4] in its separation from alcoholic preparations. Advantage was taken of its unusual solubility characteristics in developing a one-step extraction procedure in which an aliquot of rapeseed oil is diluted with petroleum ether and extracted with methanol–water (1:1). Recovery problems encountered in developing a suitable extraction procedure were overcome by running rapeseed oil standards spiked with known concentrations of denatonium benzoate in parallel with the samples. Results are calculated from peak areas generated by a computing integrator.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Waters Assoc. (Milford, MA, U.S.A.) Model 201 equipped with a 6000A pump, U6K universal injector, and a Perkin-Elmer (Coleman) Model LC-55 variable-wavelength UV detector. The chromatograph was attached to a Fisher Recordall Series 5000 (10 mV full scale) recorder and to a Perkin-Elmer M-1 computing integrator. The chromatographic column, 25 cm × 5 mm I.D., was packed in the laboratory with the aid of an HPLC slurry packing unit with Chromosorb LC-8 (obtained from Supelco, Bellefonte, PA, U.S.A.), 10 μm particle size, a chemically bonded cyano-type polar phase on microparticulate silica. A guard column, 7 cm × 2 mm I.D., containing C_{18}/Corasil 37–50 μm (Waters Assoc.), was placed immediately ahead of the chromatographic column.

Chemicals

The methanol, acetonitrile and water were each HPLC grade. The denatonium benzoate (Macfarlan Smith, Edinburgh, Great Britain) was obtained from J. H. Walker (Larchmont, NY, U.S.A.) and the rapeseed oil was of a quality typical of that imported into the U.S.A. All other chemicals were reagent grade.

Procedure for extraction of denatonium benzoate from rapeseed oil

Pipet 5.0 ml of rapeseed oil into a small separatory funnel, add 5 ml of petroleum ether and mix. Add 5.0 ml of methanol–water (1:1) and shake gently for 30 sec. Allow the layers to separate, then draw off the lower aqueous layer into a 10-ml conical centrifuge tube. Place the tube in a beaker of warm water on a hot plate (water temperature about 50°C) and apply a gentle stream of air to the top of the tube. Evaporate to dryness, washing the walls of the tube twice with a small portion of methanol as dryness is approached to concentrate the sample in the tip of the tube. Dissolve the residue in 100 μl of mobile phase (acetonitrile–water, 60:40, containing 0.01 *M* sodium chloride).

Procedure for preparation of standards

Methanol. A stock solution containing 100 ppm of denatonium benzoate in methanol was prepared. From this stock solution, standard solutions containing 0.5, 1.0, 2.0, 5.0 and 10.0 ppm denatonium benzoate in methanol were prepared. A 5.0-ml aliquot of each was evaporated to dryness in a 10-ml conical centrifuge tube as described above. Each residue was dissolved in 100 μl of mobile phase. This series of standard solutions was used to prepare a standard curve.

Spiked rapeseed oil. A stock solution containing 100 ppm of denatonium benzoate in rapeseed oil (predetermined to be blank at 210 nm by the extraction procedure described above) was prepared. Dissolution of the solid in the oil was accomplished by shaking then sonication. Working standard solutions containing 0.5, 1.0 and 2.0 ppm denatonium benzoate were prepared from the stock solution by appropriate dilution with additional rapeseed oil (available from a number of domestic suppliers). Aliquots of these standards were extracted and run in parallel with denatured rapeseed oil samples.

Procedure for liquid chromatography

The mobile phase consisted of an acetonitrile–water (60:40) mixture containing 0.01 *M* sodium chloride (sonicated under vacuum prior to use). At the start of a run, the column was equilibrated with mobile phase. The flow-rate was 1.2 ml/min. The sample injection volume was 10 μl and the column eluent was monitored in the ultraviolet at 210 nm. Denatonium benzoate eluted in about 6.7 min. At the end of each run, the column was washed with water for approximately 30 min to remove all salt.

RESULTS AND DISCUSSION

Fig. 1 shows a standard curve obtained from a series of denatonium benzoate standards prepared in methanol. This curve clearly demonstrates the linearity of the chromatographic procedure over the range of 0.5 to 10.0 ppm. Each concentration level was run in triplicate.

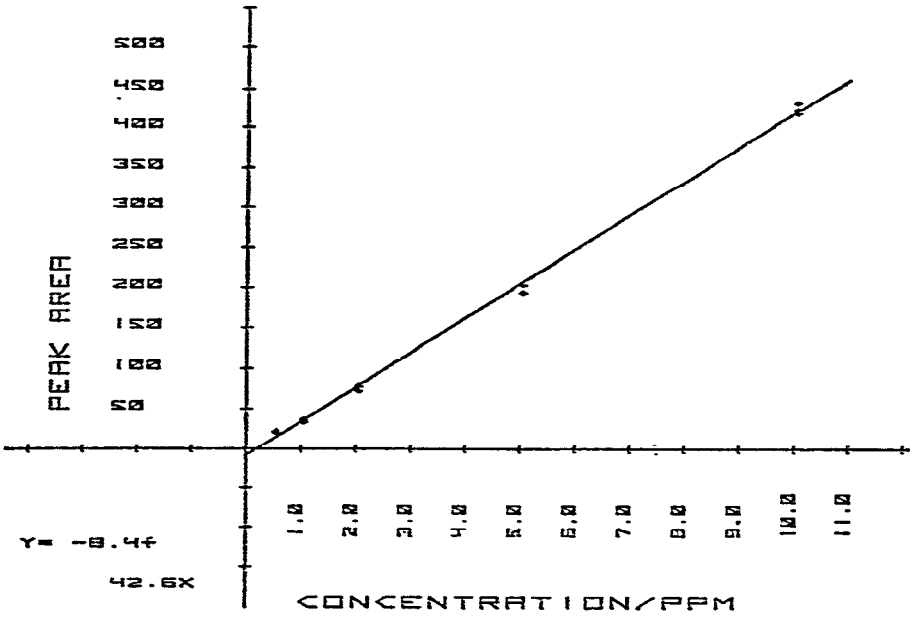

Fig. 1. Typical calibration curve obtained from denatonium benzoate standards prepared in methanol.

The results shown in Table I were obtained by comparing peak areas of rapeseed oil samples spiked with 0.5, 1.0 and 2.0 ppm denatonium benzoate with a standard curve. Though the percent recovery showed consistency, it was low.

TABLE I

RECOVERY OF DENATONIUM BENZOATE FROM SPIKED RAPESEED OIL

ppm added	*Recovery (%)*	*ppm added*	*Recovery (%)*
0 (blank)	0	2.0	68
0.5	68	2.0	67
0.5	71	2.0	80
1.0	72	2.0	72
1.0	75	2.0	72
2.0	75	2.0	80
2.0	67		

Separation

Considerable effort was expended in attempting to improve the extraction procedure. Denatonium benzoate is a quaternary amine whose structure, seen in Fig. 2, is more complex than most. Its solubility in water is 4.5% (w/v) yet it is 15 times more soluble in methanol, 7 times more soluble in chloroform and practically insoluble in ether. While attempts were made to utilize this solubility data to advantage, no extracting solvent combination tried performed better than the one ultimately selected. Slight improvement in the percent recovery was observed when the volume of the extractant was doubled or the volume or proportion of petroleum ether was increased but none of these improvements was large enough to warrent adopting a more lengthy procedure.

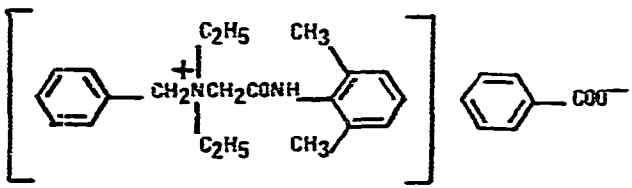

Fig. 2. Structural formula of denatonium benzoate.

In the course of this investigation, similar extractions were performed on corn oil and mineral oil spiked with comparable levels of denatonium benzoate. Following chromatography of the extracts, comparison of peak areas of these samples with the standard curve yielded even lower recoveries than for rapeseed oil suggesting that the ease of separation is matrix dependent. It is evident that an equilibrium exists between the dissociated and undissociated forms of denatonium benzoate and that these forms are not extracted with the same efficiency. To compensate for the low recovery, it was decided that comparison of peak areas of spiked rapeseed oil standards with those of denatured samples, extracted and run in parallel, offered the best solution. Commercial samples of denatured rapeseed oil gave results in the expected range. The use of a fixed loop injector in place of the U6K universal injector, where sample

volume is dependent upon the amount measured in a syringe, is recommended since it would eliminate the necessity of repetitive injections.

Because sample extracts required concentration for detection, the possibility that the residues resulting from evaporation to dryness were redissolving slowly or with difficulty was examined. Sample tubes were stoppered and allowed to stand 2–3 h following the addition of the 100 μl of mobile phase before injection into the liquid chromatograph or were mixed on a vortex mixer or in a sonicator instead of the usual practice of gentle manual mixing followed by a wait of approximately 15 min. While no increase in peak area or peak height was found as the result of these additional manipulations, it was observed that the chromatographic solutions remained stable for at least 5 days when properly stoppered.

Chromatography

A C_{18} column having octadecyl groups chemically bonded to microparticulate silica was initially tested using methanol–water containing pentanesulfonic acid as the mobile phase. This chromatography resulted in assymetrical peaks and minimal separation of denatonium benzoate from the solvent "front". In addition, the apparent efficiency of this column was greatly reduced from what previous experience indicated it should be. Modifying the mobile phase by changing its composition or pH or employing a different ion-pairing agent yielded no improvement. An amine column and a diol column (amine or diol groups chemically bonded to microparticulate silica) were each tested with a wide range of mobile phase compositions with similar results. The column ultimately selected was a cyano column whose packing consisted of cyano groups chemically bonded to microparticulate silica. We are aware of few publications describing applications of this bonded phase.

One advantage in using bonded phases is the range of polarity that they offer. With the cyano column it was observed that, in the absence of an ionic specie in the mobile phase, denatonium benzoate was retained by the stationary phase whereas the opposite was true of the C_{18} column. It was found that ionic strength was, in fact, the critical factor affecting separation. Several common salts were tried (*e.g.* potassium chloride, potassium perchlorate, sodium phosphate and sodium chloride), and sodium chloride (0.01 *M*) was selected because of its low cost and ready availability. As expected in reversed phase, increasing the proportion of water in the mobile phase increased the retention time. The 60:40 ratio of acetonitrile–water afforded complete baseline separation of denatonium benzoate from the solvent "front" while keeping run time to a minimum. Examples of typical HPLC curves are shown in Fig. 3.

In this system cetyltrimethyl ammonium bromide (cetrimide) eluted with the solvent "front" while cetylpyridinium bromide was strongly retained. The fact that, of the compounds tested, this system appeared uniquely suited only for denatonium benzoate led to abandoning a search for an internal standard. Denatonium benzoate gave maximum absorbance at 210 nm in our spectrophotometer.

Englehardt[7], in discussing chemically modified stationary phases, notes that since it is impossible to react all the surface hydroxyl (silanol) groups on the silica, the selectivity of the stationary phase is also affected by the remaining ones. Pryde and Gilbert[8] note that the mechanism of reversed-phase chromatography has not been satisfactorily worked out as yet. These authors state that retention on chemically modified supports must be by a mixture of adsorption and partition. Kirkland and

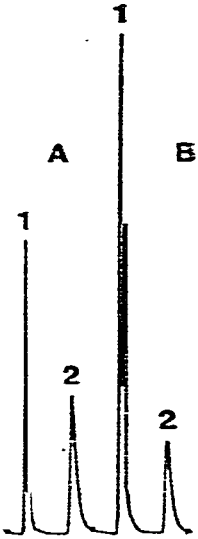

Fig. 3. Liquid chromatograms of (A) standard and (B) sample. 1 = Solvent "front"; 2 = denatonium benzoate.

De Stefano[9] state, too, that the chemically-bonded cyano phase selectively retains compounds which can readily hydrogen bond. It is possible that the chloride ion in this system serves to block those silanol sites not chemically bonded which might otherwise interact with the solute impeding its progress through the column. Salting out effects are also a possibility since it was our observation that the anion used in the mobile phase mattered little providing it was available in sufficient quantity.

We were unable to suppress completely the dissociation of the denatonium benzoate. The first peak to elute (at the solvent "front") generally contained a shoulder and sometimes appeared as two overlapping peaks. These are believed to be denatonium chloride and benzoic acid. The latter eluting peak would appear to be denatonium benzoate in undissociated form. Lowering the pH of the mobile phase to 4.0 with hydrochloric acid resulted in a 30% increase in the area of the first peak. Buffering at pH 7.0 offered no advantage over a system containing the salt alone. Experiments conducted with buffered mobile phases in the pH 4.0 to 8.0 range, the normal working range for liquid chromatographic systems, showed that it was impossible, within those confines, to convert denatonium benzoate entirely to one form or the other. The linearity observed in the standard curve when the area of the second peak (denatonium benzoate) was plotted against concentration indicated that the procedure outlined in this paper was quantitative.

CONCLUSIONS

The method presented in this paper offers a means for the rapid separation and quantitative determination of denatonium benzoate in rapeseed oil. The chromatographic system described is specific, sensitive, and affords complete separation of the compound from the solvent "front". The utility of the cyano bonded phase for separating this type of compound is clearly demonstrated.

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