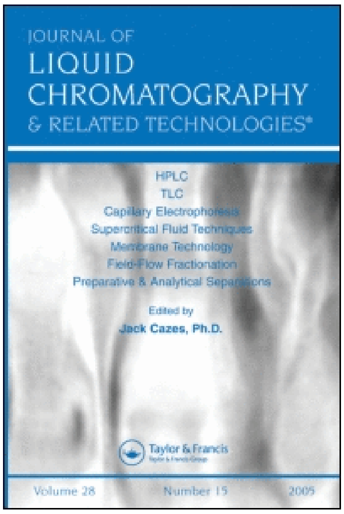

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DETERMINATION OF BITREX, QUASSIA POWDER AND SUCROSE OCTAACETATE NEXT TO DIETHYL PHTHALATE AND CAMPHOR IN SPECIALLY DENATURED ALCOHOLS BY LIQUID CHROMATOGRAPHY.

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ABSTRACT

A HPLC method for determination of the bitter principles, bitrex, quassia and sucrose octaacetate, next to other ingredients in Specially Denatured Alcohol formulations is described. The method is based on evaporation of a sample, extraction of the residue with hexane and analysis of the extracted residue on a Cyano-type column with acetonitrile-water used as the eluent. Baseline separation of compounds and satisfactory quantitation has been achieved. Samples without pretreatment can occasionally be analysed with slight modifications of the procedure.

INTRODUCTION

While the bitter substances, Bitrex, (Benzyldiethyl(2,6-xylylcarbamoylmethyl)ammonium benzoate) Quassia powder and Sucrose Octaacetate (SOA), used in several formulations of specially denatured alcohols can be, in exceptional circumstances, identified and to some extent

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quantitated by UV or IR, the spectroscopic determinations generally fail, when other strongly absorbing compounds, such as diethyl phthalate, are present. It was therefore necessary to develop a technique for separating the compounds of interest from the contaminants prior to determination. Liquid chromatography seemed to be such a technique.

Two LC methods have been described in the literature for separation and determination of Bitrex from a sometimes complex matrix of compounds (1,2); there are none, to our knowledge, for the other two denaturants. One of the methods, using a reversed phase type of a column and acetonitrile-water mixture as mobile phase seemed to have promise also for the other compounds and was therefore selected to be adapted for the general application in determining all three bitter principles simultaneously.

The low concentrations of the denaturants used in the allowed (3) formulations (see Table 1) suggested that some sort of preconcentration step might be necessary and therefore a concentration procedure was investigated. The high proportion of possibly interfering compounds called for their removal prior to LC and an extraction procedure was also desirable; the distribution properties of the main ingredients were therefore studied.

EXPERIMENTAL

Apparatus and Chromatography Conditions:

The analyses were performed using a Waters Associates Liquid Chromatography system, consisting of:

Model 6000A Solvent Delivery System
Model U6K Injector
Model SF770 Spectroflow Monitor (Schoeffel Instruments Corp.)
Model 95-8290 Honeywell Dual Pen Flat Bed Recorder.

TABLE 1

Regulation (3) Formulas Containing Bitrex and/or Quassia Powder and/or Surose Octaacetate, with Concentrations Expressed in mg/L of Sample.

Formula	Ethanol % vol	DEP*	Camphor	SOA*	Quassia	Bitrex
1 B a	95	—	—	—	140	—
1 B b	95	—	—	980	—	—
1 B c	95	—	—	420	—	7
1 F a	93	22400	400	—	110	7
1 F b	93	22400	400	210	—	—
1 S	94	11200	—	—	—	14

*DEP Diethyl Phthalate;
*SOA Sucrose Octaacetate

Column: μBondapak-CN (Waters Associates), 3.9 mm ID, 300 mm length, Stainless steel, P/N 41515, particle size 10 μm.

Hamilton Syringe, 10 μL.

Mobile Phase: Acetonitrile, (Caledon) 30% by volume, in water (distilled, for LC) (Baker Analyzed) unless otherwise indicated. The solution was degassed prior to use by sparging for 3-5 min, using pure helium.

Flow rate: 1 mL/min. (Operating range 600 - 1200 psi.) UV: 210 nm, range 0 - 0.4 A, unless otherwise indicated.

Chart speed: 8mm/min.

Rentention times of reference compounds are summarized in Table 2, for 21°C.

TABLE 2

Rentenion times: (min, and standard deviation; number of determinations in brackets)

Acetonitrile %	60	50	40	30	30, in 01-M NaCl
Bitrex	2.0±0.1 (10)	1.79±0.02 (5)	1.86±0.05 (5)	1.90±0.05 (13)	2.4±0.2 (18)
Quassia (main peaks)	3.0±0.1 (9)	3.45±0.07 (2)	4.1 (2) 4.6 (2)	5.27±0.05 (9) 6.10±0.05 (9)	5.2±0.1 6.05±0.07
SOA *	3.53±0.07 (8)	4.69±0.06 (4)	7.1 (2)	13.2±0.4 (13)	12.8±0.1
DEP *	3.74±0.07 (15)	4.76±0.05 (10)	6.6 (2)	9.8±0.1 (12)	9.7±0.2
Camphor	3.63±0.08 (6)			7.2±0.3 (2)	
Ethanol	2.1 - 3.2	2.1 - 3.2		2.5 - 3.6	2.4 - 3.6
Front peak	1.8	1.8	1.8	1.75	2.11±0.03 (20)

* SOA Sucrose Octaacetate; DEP Diethyl Phthalate

Impurities and Contaminants (Retention Times are for 30% Acetonitrile as Mobile Phase):

When samples containing large amounts of DEP were injected, signals at 1.7 - 1.9 min. were sometimes observed , ("Front Peak") possibly due to the presence of small amounts of phthalic acid in the phthalate. Peaks in the same range of retention time appeared upon injection of SOA alone, likely due to free sucrose, and were also present in samples of Quassia. Even if relatively weak, the signals might be confused with genuine Bitrex peaks from which they are only marginally resolved, and enhance the apparent Bitrex concentration.

Sample chromatograms are attached as Fig.1.

Old samples originally containing SOA exhibited, in addition to the expected peaks at 13.0 and 1.8 min., signals at 3.4, 5.6 and 8.0 min; concurrtenly the intensity of the main peak was much lower than expected to the point of total disappearance.

These peaks do not interfere with the determination of the other compounds.

Calibration:

Primary standard solutions were prepared by weighing, acurately, about 8 mg Bitrex, 100 mg Sucrose Octaacetate and 60 mg Quassia into separate 10.00 ml volumetric flasks and filling each flask up to the mark with mobile phase (30% Acetonitrile in water).

Note: The amount of SOA is close to the solubility limit under these conditions; e.g., 450 mg did not dissolve in 10 ml of this solvent. Secondary solutions of Bitrex and of Quassia were prepared by diluting to one fifth, one tenth and one twentieth of the original concentration;

Secondary solutions of SOA were prepared by diluting to one half and one fifth of the primary, using the same mobile phase as solvent.

10 µL of each solution were injected in succession, and run under the conditions specified above except that the Range (UV) of 1.0 was used for primary standard of Bitrex, and the Range of 0.1 for solutions a,b and c of Quassia; all other solutions were run at the standard Range of 0.4 A. The heights of peaks corresponding to the compounds analyzed were measured (see Table of retention times) in mm, vertically, from top of the peak to

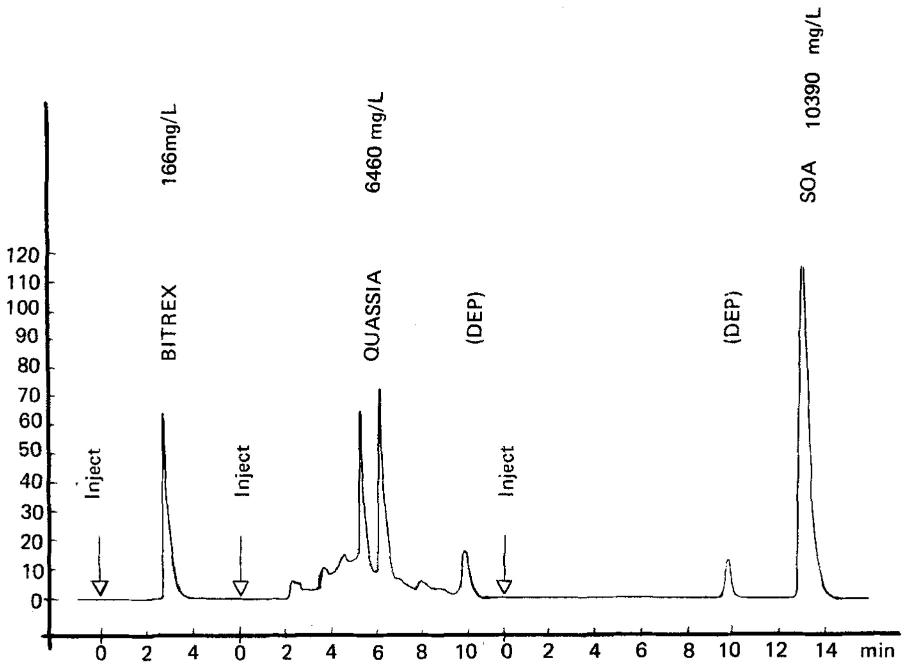

FIGURE 1

Chromatograms of Bitrex, Quassia and Sucrose Octaacetate, 10 μL, 30 % Acetonitrile in 0.01 M NaCl, detector range 0.4

the base line determined as connection between the two adjacent valleys. The heights obtained in runs at Ranges other than 0.4 were recalculated to standard 0.4 range.

Response factors were calculated by linear regression "through origin" (4,5,6) according to the equation:

$$\text{Concentration (mg/L)} = B \times \text{height (mm)}$$

The estimate of the standard error (S) is given as

$$S = (\Sigma \ (\delta y)^2 \ / \ (n-1) \ )^{\frac{1}{2}} \ \text{(mg/L)}$$

The response factors are summarized in Table 3.

TABLE 3

Response Factors for Converting Peak Heights (mm) to Concentrations (mg/L)

Compound (Concentration)	30% Acetonitrile/water			30% Acetonitrile/0.01 M NaCl		
	Factor B	Std. error S	Correl. coeff	Factor B	Std. error S	Correl. coeff
Bitrex (40 - 840 mg/L)	0.98	19	0.9991	1.60	5	0.9999
Quassia, at 5.3 m	85	66	0.989	98	18	0.999
at 6.1 m (300-6500 mg/L)	80	38	0.996	80	17	0.999
SOA (2000-20400 mg/L)	90	279	0.997	89	303	0.999

Relative standard deviation of these factors (B) is in the range of 2 - 10%. Corresponding factors for diethyl phthalate and for Camphor are 1.1 and 28 , resp., as determined from single concentration runs.

The range of relative standard error, expressed as the percentage of the standard error from the concentration limits used, is 0.6 to 48% for Bitrex, 0.3 to 22% for Quassia and 3 to 15% for Sucrose Octaacetate, the higher values corresponding to the lower ends of the concentrations.

Sample Preparation:

A/ By evaporation - dissolution.

Suitable for samples containing one or more of the bitter principles only, such as formulas 1-B. (Table 1). The sample as received (10.0 mL) was pipetted into a round glass dish (80 x 45) and evaporated to dryness from the top of a boiling water bath (about 20 min); the residue was transferred quantitatively into a 3.5 mL vial using 1-2 mL of acetone (SOA or Bitrex suspected) or methanol (Quassia suspected) and the solvent evaporated in a stream of nitrogen with slight heating (up to 40°); the residue was dissolved in 1000 µL of mobile phase (30% or more Acetonitrile in water), 10 µL of the solution injected, and the peaks identified by retention times. The concentrations of the compounds found in the sample were calculated using peak heights and the factors established in calibration.

The concentration in the original sample is taken as one tenth of the concentration so determined.

B/ By evaporation - extraction:

Suitable for samples containing diethyl phthalate, camphor and possibly other contaminants, such as in formulas 1-F and 1-S. (Table 1). The sample was evaporated and the residue transferred to a 7 mL vial as under (A) above; the residue was dissolved by shaking thoroughly for 60 seconds with 1000 µL of 30% Acetonitrile in water mobile phase and 5.0 mL hexanes (UV grade), left stand to separate, and a portion of the bottom layer used for injection into LC. Concentrations of detected bitter compounds were calculated as under (A).

While camphor was virtually removed from the mixture in this procedure, a strong signal corresponding to diethyl phthalate was still present in the chromatogram; this signal is well resolved from, and does not interfere with, the signals of compounds of interest.

C/ Direct injection:

Suitable for clean samples without contaminants; alternative to procedure (A). 20 µL sample as received (only filtered, if necessary) was injected and run at Range 0.1 A. The peak heights were measured and standardised and concentration calculated using response Factors. The concentration so determined is the concentration in the sample. When procedure (C) was used with samples containing large amounts of phthalates or similar contaminatns, the huge peak of the phthalate (9 - 12 min R.T.), and the minor components usually contained in the phthalates used, tended to interfere with some of the peaks to be measured and made the peak identification difficult; further, the contaminants tended to stay partly behind in the LC system and cluttered the subsequent runs; extensive cleaning of the column and injector was usually required in such a case.

Estimate of the Distribution Coefficient of Diethyl Phthalate.

A known amount of diethyl phthalate was shaken with 1000 μL mobile phase and 5.0 mL hexanes, the mixture left standing to separate and the bottom layer analysed by LC for diethyl phthalate as described above. The results are summarized in Table 4.

As shown, a single extraction of 1000 μL solution of the evaporation residue in mobile phase of not more than 30% acetonitrile with 5 ml hexane removes the phthalate sufficiently for subsequent LC analysis of the watery phase.

Test Runs.

Several samples were prepared by weighing to simulate working solutions obtained by procedure A and precedure B. Samples of denatured spirits were also tested following procedure C. The results are summarized in Table 5.

RESULTS AND DISCUSSION

When inorganic salts are used as constituents in the mobile phase, as suggested in the literature (1,2) a lengthy system

TABLE 4

Distribution Coefficients for Diethyl Phthalate between Mobile Phase and Hexane, at 21^{0} C

Mobile phase: (% acetonitrile)	60	50	25
Distribution coefficient, K	0.75	1.9	19

cleaning is required daily after each series of runs. To save time, most of the experiments were run without the addition of salts; no apparent deterioration of peak shapes or significant changes in retention times were observed when compared to results of experiments using sodium chloride.

The ratio 60/40 for the acetonitrile/water system provided very good separation of the three bitter substances from one another. Sharp, strong singletts were obtained for Bitrex and SOA and a multiplet for Quassia powder as corresponds to the complex nature of the latter. The total analysis time of less than 4 minutes was very appealing also. (Table 2.) However, very little or no separation was observed for SOA and diethyl phthalate, which precludes the use of this system even if most of the phthalate is removed in advance by extraction. Gradual increase of the proportion of water in the mobile system shifted the retention time of SOA sufficiently away from the signal of the phthalate, until a satisfactory separation was achieved at a ratio of 30/70, with the analysis time being still in the acceptable range of below 15 min. At the same time, the resolution between all the other compounds, including camphor and, possibly, ethanol, has improved also. The response (Peak height corresponding to unit concentration) that necessarily decreases with longer retention times due to peak broadening, remained sufficiently high for the ten-fold concentrations, and,

TABLE 5

Chromatography of Test Samples

Sample #	Composition mg/L	Procedure	30% Acetonitrile/ water		30% Acetonitrile/ 1 M NaCl	
			Found, mg/L	% of expected	Found, mg/L	% of expected
a) Compounds dissolved in mobile phase and analysed						
1	SOA: 328	A	324	99		
	DEP: 56		---	--		
2	BIT: 8.3	A			7.5, 7.7	90,93
	SOA: 520				506, 525	98,101
b) Compounds dissolved in mobile phase, extracted with hexane and analysed						
3	BIT: 8.3	B			9.3, 10.2	112,123
	QUAS: 129				108, 104	84,81
	DEP: 22400					
4	SOA: 208	B	122	59	116, 116	56,56
	DEP: 22400					
5	BIT: 16.6	B			16.5, 19.7	99,119
	DEP: 11200					
c) Compounds dissolved in ethyl alcohol (95%)						
6	QUAS: 142.5	A	148	104		
		C	140	99	150, 150	105,105
7	BIT: 7.1	C	6.8	95	5.0, 5.4	70,76

Abbreviations in the table:
BIT Bitrex
SOA Sucrose Octaacetate
QUAS Quassia powder
DEP Diethyl Phthalate

as was shown later, remained sufficient even for concentrations of the bitter compounds in samples as received (Regulation formulas), when the sensitivity setting was properly adjusted.

Addition of salt (NaCl) to the mobile phase (0.01 M) seemed to improve the separation of Bitrex from the front peak, but this advantage proved to be temporary. Repeating the injection of an appropriate mixture after about ten injections of other compounds gave a chromatogram with the first two signals strongly merged, similar to signals obtained with the mobile phase without salt. The resolution power of the column was restored after thorough washing (30 min) with water and Acetonitrile (10 min), but again only for a short time. At the same time the slight increase in retention time of Bitrex brought the peak in close proximity to the ethanol signal, so that this system could not be used for Bitrex determination in alcohols as received, if such an approach were chosen. The salt did not have any significant effect on the position, strength or resolution of signals of all the other compounds investigated. The more practical salt-free phase of 30 % acetonitrile in water is therefore recommended for routine use. When equivocal results regarding Bitrex concentration are obtained, the 0.1 M NaCl containing mixture could be used, but only after thorough conditioning of the chromtographic system.

The linearity of the responses in the ranges defined by the concentration limits of the calibration solutions calculated by linear regression and expressed as correlation coefficients, is satisfactory: 0.999 for Bitrex, 0.998 for Quassia (at 6.1 min) and 0.997 for SOA. While Bitrex and SOA give a single peak under the conditions described and there is no ambiguity as to choosing the analytical reference, Quassia powder invariably gives a multiplet of at least six discernible peaks, two of which are prominent and almost of equal intensity (5.3 and 6.1 min); either one of these two can be used for height (and concentration) measurement, when the proper corresponding factor is used.

Several samples of Quassia powder of very different provenance were analysed using the described procedure; there was no apparent variation between the chromatograms representing the different samples.

There was an opportunity to analyse samples containing SOA (Formulas 1 B type c, and 1 F, type b) that were kept in the laboratory in a closed bottle for 4 - 5 years. Invariably, the content of SOA in these solutions decreased below 50 % of the declared concentration-some had virtually a zero content -, with the concommitant emergence of new, not identified peaks, none of them interfering with the recognised signals described in Table 2. They are most likely due to solvolysis products of SOA, such as glucose tetraacetate, fructose tetraacetate, lower acetates of one of the sugars (sucrose, glucose, fructose) and possibly sucrose itself (ret. time for sucrose was found to coincide with the "front peak", i.e. 1.75 min). While this finding is not detrimental to the procedure, it raises doubts about the long term efficacy of Sucrose Octaacetate as a denaturant.

For reasons of chromatographic practicality - such as working at intermediate sensitivity with resonable baseline stability and signal strengh - it was considered advisable to increase the concentration of the sample about ten times as compared to samples as received; this would at the same time allow for removal of the bulk of ethanol, whose signal just might interfere with the closely neighbouring peaks of Bitrex on one side and Quassia on the other. Such a preconcentration is easily achieved by evaporating exactly 10 mL of sample and dissolving the residue in exactly 1 mL of a non-interfering solvent, such as the mobile phase itself, as described in the Experimental.

Clear solutions were obtained. The multiple transfer of the material, however, was reflected in incomplete recovery.

In view of the concentration ratios of diethyl phthalate to bitter substances in the appropriate formulas (Table 1) it was deemed advisable - and was born out in experiments - to remove the bulk of the phthalate, and camphor prior to LC analysis, in spite of the good separation of the compounds in question. To assess the suitability of likely solvent systems for the extraction procedure, distribution coefficients were determined by analysing the water layers for residues of the phthalate. The coefficients were found to increase dramatically when water content in the system mobile phase/hexane was increased.

The concentration of SOA is decreased in this process to about 60 % of the original, Quassia to about 80 %, whereas Bitrex concentrations seem not to be affected.

The preconcentration procedure discussed above lends itself directly to the extraction (see Experimental).

The results obtained by chromatography of the concentrates and correspondingly prepared standard mixtures, suggested that the signals are sufficiently strong to be detected in samples as received, if the injection volume is doubled and the sensitivity increased about four times. Experiments have borne this out, at least for simple formulas such as the 1 B types. The lack of sample pretreatment, however, leads to appearance of huge "ghost" peaks in several chromatograms after injections of more complex samples, such as formulas 1 F, samples of questionable purity and older samples. For these reasons, and also for the possibilty of losing the Bitrex signal in the closely following strong ethanol peak, this alternative procedure cannot be recommended for routine use; it might prove useful for quick and simple checks on occasion, particularly for Quassia or SOA analyses in the absence of large amounts of contaminants.

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