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USE OF CATION-EXCHANGE RESIN FOR THE DETECTION OF ALKYLPYRIDINES IN BEER

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SUMMARY

A method has been devised whereby trace amounts of certain basicscompounds, such as pyridines, may be detected and semi-quantified in beer in the resence of an excess of other flavour constituents including pyrazines. The method involves steam distillation of beer under reduced pressure and subsequent passage of the distillate through a column of weakly acidic Zerolit cation-exchange resin. The resin is eluted with aqueous sodium chloride, the eluate extracted with organic solvent and the concentrated extract analysed by gas chromatography coupled with mass spectrometry. Using this technique with multiple ion detection, a series of alkylpyridines was readily detectable in beers and worts at levels below 1 ppb*.

INTRODUCTION

Many investigators have recognised the important contribution which certain volatile basic compounds can make towards the aroma and flavour of some beers[1–5]. However, of the components which have been chemically characterised in basic extracts of beers, pyrazines are the group which have received most attention[3–5]. This is presumably because they are the predominant class of compounds occurring in basic extracts of beer obtained using techniques based upon solvent extraction[3–5], and because they are well-known to be a highly flavour-potent group of substances[6]. Other volatile basic constituents of beer which have been identified include amines[1], amides[5] and traces of a small number of pyridines[2,4,5] and thiazoles[4,5]. However, detection of the latter two groups of compounds in beer is very difficult because they tend to be masked by the relative excess of pyrazines which are present. This investigation set out to devise a method for detecting trace volatile basic compounds, other than pyrazines, which occur in beer.

* Throughout this article, the American billion (10^9) is meant.

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EXPERIMENTAL

Reagents and standards

Zerolit 236 SRC 41 cation-exchange resin (7 g dry weight) was washed with 0.6 *M* hydrochloric acid (100 ml) and deionised water (200 ml) prior to use.

Pentane (Koch-Light, Colnbrook, Great Britain) and diethyl ether (BDH, Poole, Great Britain) were both redistilled prior to use. Antifoam was a solution of 10% silicone DC antifoam RD emulsion (Hopkin and Williams, Chadwick Heath, Great Britain) in water. Authentic beer flavour constituents were mostly obtained from commercial suppliers, and were homogeneous according to analysis by GC. The amino acid ethyl esters[7] were obtained from a commercial supplier or were synthesised according to a literature procedure. A sample of 2,3-dimethylpyridine was the generous gift of Dr. M. Novotný of Indiana University, Bloomington, IN, U.S.A.

No interfering pyridines were detected in any of the reagents described above.

Preparation of wort and beer

Wort and beer were prepared on the pilot scale using a standard procedure[8].

Preparation of basic extracts of wort and beer

Wort or beer (4 l) containing antifoam solution (10 ml) was vacuum steam distilled at 25°C and 0.02 mmHg pressure, using the method of Pickett *et al.*[9]. The distillate was then passed through a column of Zerolit 236 SRC 41 cation-exchange resin (110 × 12 mm, 7 g dry weight) at *ca.* 7 ml/min. The resin was washed with deionised water (200 ml) and subsequently eluted with 2 *M* aqueous sodium chloride (230 ml). The pH of the eluate was adjusted to *ca.* 8.5 by addition of 2.5 *M* aqueous sodium hydroxide, and the eluate then continuously extracted for 6.5 h with a 1:2 mixture of pentane and diethyl ether (40 ml). After drying over anhydrous sodium sulphate (5 g), the organic extract was concentrated to 0.5 ml, by gentle warming on a water bath (40°C) using the technique of Junk *et al.*[10], spiked with 3 μl of a 1% solution of ethyl octanoate in ethanol, further concentrated to 0.2 ml and finally examined by gas chromatography (GC) and GC–mass spectrometry (MS).

Recoveries of authentic compounds from 4% aqueous ethanol using the Zerolit procedure

Dilute solutions, containing mixtures of authentic compounds (1 ppm each) in 4% aqueous ethanol, were passed through a column of Zerolit 236 SRC 41 resin (110 × 12 mm). The compounds were eluted from the resin and concentrated using the same technique as described above for beer extracts. Concentrates were then examined by GC (using a conventional packed column) and peak heights (obtained by flame-ionisation detection) compared to those obtained for standard mixtures of of the appropriate compounds.

Instrumental analyses

Gas chromatography. GC of beer extracts was carried out using a support-coated open tubular column coated with Carbowax 20M[7].

GC of mixtures of authentic beer flavour compounds was carried out using a Pye GCV gas chromatograph, equipped with a linear temperature programmer and synchronous flame-ionisation, flame-photometric (394 nm filter) and alkali flame-

ionisation detection. The GC column, 2.8 m × 4 mm I.D. glass packed with 20% Carbowax 20M on Chromosorb W AW DMCS (80–100 mesh), was operated with a nitrogen carrier gas flow-rate of 78 ml/min and a temperature programme of 50–200°C at 3°C/min.

Combined gas chromatography–mass spectrometry. This was carried out using a Finnigan 1020 GC–MS system. Chromatographic separations were performed with a 50 m × 0.3 mm I.D. glass capillary wall-coated open tubular column coated with Carbowax 20M (GC² Chromatography, Northwich, Great Britain). Helium (CP grade, B.O.C., London, Great Britain) was used as carrier gas at a linear flow-rate of 26.5 cm/sec, and the oven temperature programme employed was 60°C for 5 min followed by an increase of 3°C/min to 200°C.

Effluent from the capillary column was passed directly into the source of the mass spectrometer via a heated 0.1 mm I.D. glass capillary transfer line. The source temperature was 80°C and the transfer line was maintained at 230°C. Mass spectra, measured with an ionisation energy of 70 eV, were either scanned from *m/e* 45 to 250 every 1 sec, or were scanned in the multiple ion detection (MID) mode as described below. All data acquired were stored on disk for later recall.

RESULTS AND DISCUSSION

In preliminary work basic extracts of beers were prepared using a variation of the commonly employed solvent extraction technique. The pH of the beer was adjusted to *ca.* 8.5, prior to steam distillation under reduced pressure at ambient temperature according to the method of Pickett *et al.*[9]. The distillate (after pH adjustment to *ca.* 8.5) was then continuously extracted with organic solvent, followed by by acid extraction of the organic phase. After washing of the acid phase and subsequent pH adjustment to *ca.* 8.5 it was re-extracted with organic solvent. The majority of the solvent (pentane–diethyl ether, 1:2) was then removed by gentle warming on a water bath (40°C), using the technique of Junk *et al.*[10]. Examination by GC–MS, of extracts produced in this way, confirmed that pyrazines were the major class of volatile basic compound present. Several pyridines (of molecular weights 93 and 107) could be detected in extracts prepared from a commercial ale and a commercial stout, but the mass spectra were not of sufficiently high quality to allow the exact nature of the isomers to be determined. It was noticeable that pyridines were detected only in those sections of the chromatogram which were relatively free of pyrazines and other major components. Subsequently beers with known additions of both pyrazines and pyridines were extracted and concentrated. Recoveries of the added compounds were actually found to be rather higher for pyridines (*ca.* 60–90%) than for pyrazines (*ca.* 40–70%), showing that the solvent extraction method itself was not responsible for the relative levels of pyrazines and pyridines noted in beer extracts.

Peppard and Douse[11] recently reported the use of Amberlite XAD-2 resin in preparing concentrates of beer flavour compounds. The technique was therefore investigated in the present studies connected with basic beer constituents. Using mixtures of authentic compounds (0.5 ppm each) in 4% aqueous ethanol, the recoveries of 2,5- and 2,3-dimethylpyrazines and 2-, 2,4- and 2,4,6-methylpyridines were all poor (8–20%) as was the recovery of thiazole (2%). The recoveries of more heavily substituted compounds using the resin were considerably better, *e.g.* 2-methyl-3-

acetylpyrazine (68%), 6,7-dihydro-2,5-dimethyl-5H-cyclopentapyrazine (83%) and 2-ethyl-4,5-dimethylthiazole (84%), but the method clearly was not as efficient for concentrating the lower molecular weight pyridines from beer as the previously described solvent extraction technique and so was not investigated further.

TABLE I

pKa VALUES OF SOME COMMON PYRAZINE, PYRIDINE AND THIAZOLE DERIVATIVES[12,13]

Compound	*pK_a Value*
Pyrazine	0.65
2-Methylpyrazine	1.45
2,5-Dimethylpyrazine	1.85
Pyridine	5.20
2-Methylpyridine	5.95
2-Ethylpyridine	5.90
2,5-Dimethylpyridine	6.45
Thiazole	2.44

Pyrazines, being weaker bases than either pyridines or thiazoles, generally have lower pK_a values, as indicated by the examples given in Table I. The possibility of exploiting such large differences in basicity in order to separate these classes of compounds was therefore investigated. In the first instance the strongly acidic cation-exchange resin Dowex 50W-X8 and the weakly acidic cation-exchange resin Zerolit 236 SRC 41 were tested using mixtures of authentic compounds (1 ppm each) in 4% aqueous ethanol and eluting the resin columns with 2 *M* aqueous sodium chloride. The compounds used included pyrazines, pyridines, amines and thiazoles as well as several neutral and acidic volatile beer flavour constituents.

Some typical recoveries achieved using the Dowex resin are given in Table II. All of the basic compounds tested were recovered to some extent and were thus separated from non-basic compounds. However, recoveries of certain basic compounds were extremely poor, and in any case the results indicated that the resin was not suitable for separating pyridines and/or thiazoles from pyrazines.

TABLE II

RECOVERIES OF COMPOUNDS FROM 4% AQUEOUS ETHANOL USING DOWEX 50W-X8 RESIN (62 × 9 mm)

Compound	*Recovery (%)*
2,5-Dimethylpyrazine	41
2,3-Dimethylpyrazine	44
Thiazole	44
2-Butyl-4,5-dimethylthiazole	19
2-Methylpyridine	57
2-Ethylpyridine	55
Ethyl octanoate	0
l-Carvone	0

TABLE III

RECOVERIES OF COMPOUNDS FROM 4% AQUEOUS ETHANOL USING ZEROLIT 236 SRC 41 (110 × 12 mm)

Compound	*Recovery (%)*
2-Methylpyrazine	0
2,3-Dimethylpyrazine	0
Thiazole	0
4-Methylthiazole	4
2-Isobutylthiazole	25
4-Ethyl-5-methylthiazole	68
2-Isopropyl-4-methylthiazole	67
2-Butyl-4-methylthiazole	62
2-Butyl-4,5-dimethylthiazole	56
Pyridine	64
2-Methylpyridine	53
2-Ethylpyridine	88
2,4-Dimethylpyridine	80
2,4,6-Trimethylpyridine	87
Ethyl valine	70
Ethyl isoleucine	75
2-Acetylpyrrole	0
2-Acetylfuran	0
2-Acetylthiophene	0
Isoamyl acetate	0
Ethyl octanoate	0
Isoamyl alcohol	0
2-Phenylethanol	0
l-Carvone	0
Octanoic acid	0

Table III shows some recoveries achieved in similar experiments using the Zerolit resin. In contrast to the results obtained using the Dowex resin, the results indicated in Table III clearly show that the Zerolit resin may be successfully used to separate pyridines and certain alkylthiazoles from pyrazines. Experiments in which the Zerolit resin was eluted successively with several batches of 2 *M* aqueous sodium chloride revealed that, whilst pyridines were relatively easily eluted from the resin under these conditions, some of the thiazoles were bound more strongly to the resin and thus required a much larger volume of eluent.

The potential use of the Zerolit resin for concentrating preferentially, small quantities of pyridines and certain thiazoles from an aqueous medium containing several per cent of ethanol as well as relatively large amounts of many other volatile flavour constituents including pyrazines, was therefore established. A method for concentrating such pyridines and thiazoles from beer, based on the use of the resin, was thus designed (see Experimental) and subsequently tested.

A commercial stout was chosen for the initial study, since this type of beer generally has high levels of nitrogen-containing heterocyclic compounds. Examination of the extract by GC, employing the use of a capillary column, revealed it to be extremely complex in composition although subsequent analysis by GC–MS showed neither pyridines nor thiazoles to be amongst the major components present. However,

application of the technique of mass fragmentography[14] did reveal a series of pyridines to be present in the extract at relatively low levels. These included pyridine itself, three compounds of molecular weight 93 (2-, 3- and 4-methylpyridines) and nine compounds of molecular weight 107 (2-, 3- and 4-ethyl- and 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dimethylpyridines). Whilst some of these compounds have distinctive mass spectra which allow tentative structural assignment to be made, the majority have spectra which are rather similar[15,16]. However, employing the technique of peak enhancement with authentic compounds, positive structural assignment was made in all cases. Other compounds detected in the basic extract of stout include a group of amino acid ethyl esters, namely ethyl valine, ethyl leucine and ethyl isoleucine, which were identified for the first time in beer[7]. There were also indications that the extract contained a series of aliphatic amines, a class of compounds which have previously been detected in beer[1]. However, mass fragmentography confirmed the expected absence from the extract of several alkylpyrazines which had previously been readily detected in the same stout using the solvent extraction procedure mentioned above. Application of mass fragmentography, using ions characteristic of alkylthiazoles[17], also failed to reveal the presence of any of this class of compound in the stout extract. Harding *et al.*[18] have detected a series of ten thiazoles in roasted barley, which is one of the raw materials used in brewing stouts as well as some ales. However, the absence of these compounds in beer extracts prepared in the present studies is to be expected at least for the simplest alkylthiazoles which, as pointed out above, are very poorly retained by the Zerolit ion-exchange resin; presumably the more highly substituted thiazoles do not survive the brewing process in amounts sufficient for detection by this method.

When basic extracts of a series of other beers (including ales and a lager) were prepared and analysed in the same way, some pyridines were detected but these were generally at much lower levels than had been detected in the extract from the commercial stout. It was apparent that in some of these extracts the compounds, if present at all, must be at levels well below the detection limit of the method. It was therefore decided to re-examine all of the extracts prepared using the much more highly sensitive method of MID[14]. Thus effluent from the capillary column was scanned (at 1-sec intervals) for components having mass spectra exhibiting ions of *m/e* 92, 106 and 107 (residence time per ion per scan = 0.13 sec) and *m/e* 101 and 127 (residence time per ion per scan = 0.01 sec). In this way ethyl- and dimethylpyridines (whose identities were confirmed by means of retention time and *m/e* 92:106:107 pattern) were quantified by measuring their *m/e* 107 response relative to the *m/e* 101 response of ethyl octanoate which was added to each sample as an internal standard. Relative response factors for the pyridines were determined by chromatographing a mixture containing 1 ppm each of the ethyl- and dimethylpyridines and again using MID with quantification based on measurement of the *m/e* 107 peak areas. Table IV lists the relative levels of ethyl- and dimethylpyridines which were measured in the range of commercial and experimental beers examined. Fig. 1 shows a chromatogram, reconstructed from the *m/e* 107 ion current, obtained from a basic extract of the commercial stout to which had been added 1, 0.5 and 0.1 ppb, respectively, of 2,5-dimethylpyridine, 4-ethylpyridine and 3,5-dimethylpyridine. The magnitudes of the increases in sizes of the responses for these three compounds indicate that all of the pyridines listed in Table IV were present in the untreated stout at levels below 1 ppb,

TABLE IV

RELATIVE LEVELS OF ETHYL- AND DIMETHYLPYRIDINES OCCURRING IN A RANGE OF BEERS

The figures in this table refer to corrected peak areas, the highest level being arbitrarily set to 1000. n.d. = not detected.

Pyridine	*Beer*			
	Commercial stout	*Experimental beer**	*Commercial ale*	*Commercial lager*
2,6-Dimethyl	112	29	31	1
2-Ethyl	93	3	1	0.3
2,5-Dimethyl	143	15	1	n.d.
2,4-Dimethyl	302	134	11	0.6
3-Ethyl	1000	74	36	2
4-Ethyl	120	26	n.d.	n.d.
3,5-Dimethyl	26	4	n.d.	n.d.
3,4-Dimethyl	797	196	5	n.d.

* Beer brewed with a grist containing 30% crystal malt.

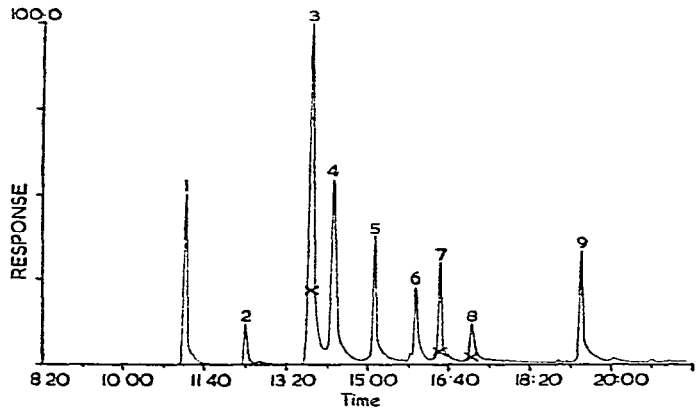

Fig. 1. Section of *m/e* 107 ion current chromatogram of extract from commercial stout spiked with alkylpyridines. Peaks: 1 = 2,6-dimethylpyridine; 2 = 2-ethylpyridine; 3 = 2,5-dimethylpyridine; 4 = 2,4-dimethylpyridine; 5 = 2,3-dimethylpyridine; 6 = 3-ethylpyridine; 7 = 4-ethylpyridine; 8 = 3,5-dimethylpyridine; 9 = 3,4-dimethylpyridine. X signifies height of peaks from beer without additions.

and in the other beers at even lower levels, becoming barely detectable in the lager. In the present studies the presence of alkylpyridines in wort prior to fermentation was also confirmed, similar levels being detected in an experimental beer (see Table IV) and the wort from which the beer was prepared.

Alkylpyridines have been detected in a wide range of food products, including whisky[19], cooked rice[20], wheaten bread[21], roasted lamb fat[22], black tea[23], Soy sauce[24], roasted cocoa[25], etc. A limited number of alkylpyridines have also been detected in roasted barley[18], but the majority of identifications reported in this case were tentative only. In addition to the occurrence, in food products, of alkylpyridines derived from natural precursors, these substances are sometimes deliberately added as flavouring

agents, often at levels well in excess of 1 ppm[26–28]. The method described here could well be applicable to the study of food products other than beer which contain the products of non-enzymic browning.

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