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Detection of Irradiation Treatment of Poultry Meat using the *Limulus* Amoebocyte Lysate Test in Conjunction with a Gram Negative Bacterial Count

Collaborative Trial

Susan Scotter,* Karen Beardwood and Roger Wood

Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, Norwich Research Park, Colney, Norwich, NR4 7UQ,.

* Author to whom correspondence should be addressed

The results of a collaborative trial of a microbiological screening test using the Limulus amoebocyte lysate test in conjunction with a Gram negative bacteria count (LAL/GNB) for identifying suspect irradiated poultry are reported. Data from this trial demonstrate that the LAL/GNB test can be used with a high degree of success to screen poultry samples for an abnormal microbiological profile which, in the absence of any visible signs of processing, is indicative of irradiation treatment.

For the interpretation of results, the use of threshold levels for LPS titre and GNB count yielded 100% of unirradiated samples and 80-94% of irradiated samples (depending on sample type and irradiation dose) correctly identified. However, an improved scheme for the interpretation of results from the LAL and GNB tests has been devised and is recommended. When the improved scheme was applied to data from this trial, it resulted in 100% of unirradiated samples and 82-97% of irradiated samples being correctly identified by participants.

No mis-identifications were reported, however, some results were reported as "inconclusive" when very low LPS titres were obtained with some irradiated samples. These low titres are believed to be due to sample variation rather than any effect of low dose irradiation on the LPS molecule. The precision of both the GNB count and the LAL assay was poor but this was attributed to the inherent microbiological variation of real food samples which were used in this trial.

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Introduction

The value of food irradiation for improving the safety and quality of foodstuffs by reducing pathogenic micro-organisms in foods, controlling parasite and insect infestation and the ripening and sprouting of fruits and vegetables is well recognised [1, 2, 3]. Food irradiation facilities now exist in over 20 countries including 6 Member States of the European Community (Belgium, Denmark, France, Germany, Netherlands and UK). As part of the general EU harmonisation programme and the completion of the Single Market, the European Commission is attempting to harmonise legislation governing the production of and trade in food treated by ionising radiation. To this end a proposal for a Directive on food irradiation has been developed[4, 5] and is being discussed. Within the UK, food irradiation is permitted for a limited number of foods under the "Food (Control of Irradiation) Regulations 1990"[6].

It is important that methods are available to determine whether foodstuffs or ingredients have been irradiated. Much work on the development of suitable detection methods has already been carried out [7, 8] and the former Community Bureau of Reference (BCR), part of the Commission of the EU, has been particularly active in this field. The Ministry of Agriculture, Fisheries and Food (MAFF) has also supported the development of methods for the detection of irradiated foods. Methods and their performance characteristics for the Detection of Irradiated Herbs and Spices (V27)[9], Detection of Irradiated Meats which Contain Bone Fragments Using ESR Spectroscopy (V28)[10] and Detection of Irradiated Poultry Meat using the *Limulus* Amoebocyte LysateTest in Conjunction with a Gram Negative Bacterial Count (V29)[11], which is based on the procedure being reported here, have been published in the MAFF Validated Methods Series.

There are two types of methods for the detection of irradiated foods; chemical procedures (e.g. thermoluminescence, ESR and cyclobutanones) which detect irradiation unambiguously and microbiological procedures which are used for screening.Two microbiological procedures have been investigated as possible screening methods for identifying foods suspected of being irradiated. These are the Direct Epifluorescence Filter Technique (DEFT) used in conjunction with an aerobic plate count (APC) and the *Limulus* amoebocyte lysate test (LAL) used in conjunction with a Gram negative bacterial count (GNB). The former has been described by Betts *et al.*[12] and has been validated by collaborative trial [8].

LAL/GNB Procedure

The principles and scope of application of the LAL/GNB test, applied by the MAFF Food Science Laboratory, Norwich, have been described by Scotter *et al.*[13]. The test was developed as a screen to detect an abnormal microbiological profile of, in particular, proteinaceous foods such as poultry meat where the contaminating flora are predominantly Gram negative. The LAL test is a semi-quantitative assay for measuring bacterial lipopolysaccharide (LPS, expressed as endotoxin units, EU) which is present in the cell walls of all Gram negative bacteria[14, 15, 16]. The LPS molecule is resistant to heating and has been shown to survive irradiation treatment. By using this test in conjunction with a selective count of viable GNB using a medium described by Phillips *et al* [17], the proportion of viable to non-viable GNB in a sample can be determined. A high LAL titre obtained in a foodstuff in the absence of viable GNB is indicative of the presence of a large population of non-viable bacteria; in the absence of any visible signs of processing of the food, irradiation treatment may be suspected.

This paper reports the results of a collaborative trial of the LAL/GNB method for detecting irradiated chicken organised in the U.K in 1993 by the MAFF Food Science Laboratory, Norwich.

Collaborative Trial Organisation

Methods

The methods for the enumeration of GNB and the determination of LPS titres used by the trial participants have been previously published [11]. A summary of procedures is shown in Fig. 1.

Participants

Twenty UK laboratories asked to participate in the trial (16 Public Analyst laboratories, 3 Public Health laboratories and the MAFF Food Science Laboratory, Norwich) and received samples. Three laboratories returned results which could not be utilised later.

Samples

A single batch of boneless chicken breasts with skin and a single batch of chicken breast fillets were supplied by a commercial poultry processor. Within 24h of slaughter, both batches of chicken were transported from the processing plant, under refrigeration conditions (<5°C), to a commercial irradiation facility. The batch of chicken breasts with skin were sub-divided; one third of the batch were retained as control (unirradiated) samples; one third were

irradiated at an overall average dose of 2.5 kGy and the remainder irradiated at 5kGy. The skinless chicken breast fillets were sub-divided into two. Half were retained as controls and the remaining half were irradiated at 2.5kGy. Samples were irradiated using a Cobalt 60 source. Amber perspex dosimeters (Harwell) were used to estimate the dose received by measuring spectrophotometrically a change in absorbance at 530 nm. Corresponding doses were obtained by comparison with a calibration curve provided by the National Physical Laboratory, Teddington.

All chicken pieces were randomly coded and packaged in insulated containers under chill conditions. They were then delivered by overnight carrier to arrive at participating laboratories the following morning. Participants were instructed to commence the analysis immediately upon arrival of the samples.

Each participant received 10 samples for analysis comprising control samples of chicken breasts with and without skin; chicken breasts with skin irradiated at 2.5 and 5 kGy and skinless breasts irradiated at 2.5kGy. All samples were dispatched randomly coded as blind 'pairs'. Participants were asked to examine each sample once only and report the results obtained from the LAL test and enumeration of GNB. From these results the participants were asked to indicate whether they considered the sample had been irradiated having been given guidance in the interpretation of results.

Instructions on interpretation or results

Participants were asked to classify whether a sample had been irradiated or not according to the following scheme:

a) samples with endotoxin concentration $>\log_{10}$ 1.7 EU/g and GNB count $>\log_{10}$ 3.5 cfu/g show a **normal microbiological profile** and are not irradiated. Such results were to be reported as "not irradiated".
b) samples whose endotoxin concentrations are $>\log_{10}$ 1.7 EU/g but have $<\log_{10}$ 2.0 GNB show an **abnormal microbiological profile** which is indicative of possible irradiation treatment. Such results were to be reported as "irradiated".
c) samples identified as having endotoxin concentrations $<\log_{10}$ 1.7 EU/g and GNB count $<\log_{10}$ 3.5 cfu/g have an inconclusive profile. Such results were to be reported as "Inconclusive".
When used in practice, samples which are reported "irradiated" by the procedure would be further investigated using chemical procedures.

Results

The results obtained by 17 of the participants for all samples tested are shown in Tables 1 - 5. Three of the participants did not follow the prescribed protocol or had exceptional difficulties with the LAL test.

Statistical analysis of the results

Because of the qualitative nature of the screening test, no statistical analysis was carried out on the results. However, estimates of the performance characteristics for both the LAL test and GNB count were calculated. Data from these methods were transformed to a $\log_{10}$ basis and then normal collaborative trial statistical procedures were carried out [(18)]. The results obtained are also given in Tables 1 - 5 and summarised in Table 6.

Discussion

Interpretation of results

Unirradiated chicken portions with skin used in this trial had a mean GNB count of $\log_{10}$ 5.96 (sd 0.53) and a mean endotoxin level of $\log_{10}$ 3.35 (sd 0.82). The skinless breast portions had similar counts of $\log_{10}$ 5.42 (sd 0.25) and $\log_{10}$ 3.07 (sd 0.78) for GNB and EUs' respectively. Irradiation of both portion types at 2.5 kGy resulted in the reduction of GNB to undetectable levels, even allowing for a period of cell resuscitation using a solid repair method [(19)]; endotoxin levels remained relatively unaffected.

Using the assigned values for the interpretation of results (not including the results of laboratories who did not interpret the results as instructed), all of the unirradiated chicken portions with skin were correctly identified by the 17 laboratories (Table 1). Of the portions with skin irradiated at 2.5 kGy, 94% were correctly identified as irradiated, with 6% being classified as "inconclusive"; of those irradiated at 5 kGy, 86% were correctly identified with 14% being classified as "inconclusive".

All the unirradiated skinless portions were correctly identified by trial participants (Table 4). Of the skinless portions irradiated at 2.5 kGy, 80% were correctly identified. An "inconclusive" profile was reported for the remaining 20% of samples.

No participants reported any major difficulties in carrying out either the LAL test or GNB count even although some participants were relatively unfamiliar with the microtitre format of the former. Several participants commented that counting colonies on the GNB medium was laborious due to their tendency to be very small.

Use of an alternative scheme for interpretation of results

Because of the potential difficulties associated with setting threshold values for GNB and LPS titres with respect to the microbiological variation often encountered with real food samples, an alternative scheme for interpretation of results has been devised. In this scheme, the $\log_{10}$ GNB value is subtracted from the $\log_{10}$ EU value; where $\log_{10}$ EU - $\log_{10}$ GNB <0, the sample is not irradiated; where $\log_{10}$ EU - $\log_{10}$GNB >0 then the sample should be suspected of being irradiated. It should be noted that where Gram negative bacteria are reported as "not detected", for the purposes of calculating EU - GNB, $\log_{10}$ GNB is assigned an arbitrary value of 0. When a low EU titre e.g. <$\log_{10}$ 2.0 and very low levels of GNB are obtained (< $\log_{10}$ 1.0), the result must be considered inconclusive as it is not possible to differentiate between very low levels of microbial contamination in the sample e.g. very high quality chicken, or irradiation treatment.

If the participants in this trial had been instructed to interpret the results according to the alternative scheme, then 100% of all unirradiated samples, 97% of chicken with skin irradiated at 2.5 kGy, 82% of those irradiated at 5 kGy and 88% of the skinless fillets irradiated at 2.5 kGy would have been correctly identified. All other samples would have been reported as "inconclusive" due to the very low EU titres obtained.

Precision characteristics of methods

Seventeen of the participating laboratories provided results which were used to estimate the precision (repeatability and reproducibility) of the two methods. Repeatability and reproducibility were calculated for the GNB count for the unirradiated chicken portions only. For the LAL assay, these values were calculated for both irradiated and unirradiated portions (Table 6).

The precision of the LAL and GNB tests determined from data from this trial was poor. Published data[20] on the performance of the LAL test with liquid egg samples reported r = 0.25 for egg samples with and EU content of $\log_{10}$ 4.25 and R= 1.0 for egg samples with EU content between $\log_{10}$ 3.0 to 4.25. It should be noted that according to collaborative trial protocol, each chicken portion was required to be examined only once. Although the participants received "pairs" of portions of each sample type, it was not possible to supply identical duplicates and thus the variation in microbiological quality between portions was reflected in a greater apparent imprecision than would have been observed if identical portions could have been examined.

Conclusion

Data from this collaborative trial of the LAL/GNB method for detecting irradiated poultry indicate that this test can be used to screen a variety of poultry samples for an abnormal microbiological profile with a high degree of success (82-100% correct identification rate). Such a test will enable enforcement laboratories to identify, cheaply and rapidly, a minority of samples which will require examination by more elaborate methods such as electron spin resonance spectroscopy[21]. The use of threshold values assigned for the LAL and GNB tests, against which results are interpreted, is not recommended as this can lead to possible mis-identification of samples due to the high degree of variability in microbial flora present in food samples. By using the alternative scheme where the difference between LPS titre and GNB count is calculated, mis-identifications, due to assigning inappropriate threshold values for EU's and GNB are less likely to occur. However, the microbiological profiles of some samples may still be inconclusive particularly where samples are of a very high microbiological quality.

Some participants reported difficulties in counting colonies on the Gram negative medium due to their small size. An alternative medium based on the selective agent monensin [22] has been investigated as a potential replacement for the nisin, crystal violet, penicillin agar of Phillips *et al* currently used to enumerate total GNB. This medium (commercial milk agar) supplemented with 35 mg/l sodium monensin (Sigma), demonstrates a high recovery rate and produces larger colonies making counting easier (unpublished data). This formulation has not yet been subjected to inter-laboratory study.

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Table 1

Log_{10} GNB and Log_{10} EU per g of skin from blind duplicate samples of unirradiated chicken portions.

Laboratory	GNB/g	LAL Titre	EU/g	Irradiated (Y/N)*
1	5.41;5.28	2.25; 1.0	3.33;2.08	n;n
2	7.18;5.50	2.75; 1.75	3.83;2.83	n;n
3	5.88;7.18	2.25; 2.25	3.33;3.33	n;n
4	6.68;7.04	2.5 ; 1.0	3.58;2.08	n;n
5	6.14;6.24	3.0 ; 3.5	4.08;4.58	n;n
6	6.41;6.91	3.0 ; 3.5	4.08;4.58	n;n
7	5.34;5.46	1.5; 1.25	2.58;2.33	n;n
8	6.30;7.04	4.0; 4.0	5.08;5.08	n;n
9	5.04;4.83	1.75; 3.75	2.83;4.83	n;n
10	6.11;5.70	2.25; 1.5	3.33;2.58	n;n
11	5.91;6.14	0.75; 3.5	1.83;4.58	n;n
12	5.46;5.46	1.5; 1.5	2.58;2.58	n;n
13	5.85;6.01	3.0; 2.25	4.08;3.33	n;n
14	5.41;5.70	1.0; 0.5	2.08;1.58	n;n
15	5.44;6.05	1.5; 2.5	2.58;3.58	n;n
16	6.83;5.08	3.0; 2.25	4.08;3.33	n;n
17	5.74;5.98	2.25; 2.5	3.33;3.58	n;n
x	5.96		3.35	
SD	0.53		0.82	
RSD%	8.94		24.44	
SD_r	0.52		0.62	
RSD_r%	8.79		18.59	
r	1.47		1.74	
SD_R	0.65		0.93	
RSD_R%	10.89		27.75	
R	1.82		2.60	

Table 2

Log_{10} GNB and Log_{10} EU per g of skin from blind duplicate samples of chicken portions irradiated at 2.5 kGy.

Laboratory	GNB/g	LAL Titre	EU/g	Irradiated (Y/N)
1	ND;ND	3.75; 2.25	4.83; 3.32	y;y
2	ND;ND	2.5; 2.5	3.58; 3.58	y;y
3	<3.48; ND	0; 1.0	ND[a] ; 2.08	i;y
4	ND;ND	0.25; 2.25	1.33; 3.33	i;y
5	ND;ND	3.0; 3.5	4.08; 4.58	y;y
6	<3.48; <3.48	2.5;; 3.5	3.58; 4.58	y;y
7	ND;ND	2.5; 1.0	3.58; 2.08	y;y
8	ND;ND	3.5; 2,75	4.58; 3.83	y;y
9	ND; <3.48	2.5; 1.75	3.58; 2.83	y;y
10	ND;ND	3.25; 1.5	4.33; 2.58	y;y
11	<3.48; ND	2.5; 1.5	3.58; 2.58	i[b];i[b]
12	ND;ND	2.25; 3.0	3.33; 4.08	y;y
13	ND;ND	2.5; 2.0	3.58; 3.08	y;y
14	ND;ND	1.25; 1.0	3.33; 2.08	y;y
15	ND;ND	2.5; 3.25	3.58; 4.33	y;y
16	ND;ND	1.5; 2.25	2.58; 3.33	y;y
17	ND;ND	3.0; 2.25	4.08; 3.33	y;y

x	Insufficient data	3.45
SD		0.60
RSD%		17.42
SD_r		0.76
RSD_r%		21.88
r		2.12
SD_R		0.80
RSD_R%		23.30
R		2.25

Table 3

Log_{10} GNB and Log_{10} EU per g of skin from blind duplicate chicken portions irradiated at 5 kGy.

Laboratory	GNB/g	LAL Titre	EU/g	Irradiated (Y/N)
1	ND;ND	1.75; 2.0	2.83; 3.08	y;y
2	<3.48;ND	2.25; 1.0	3.33; 2.08	i[b];i[b]
3	<3.48; ND	0.25; 1.25	1.33; 2.33	i;y
4	ND;ND	0.25; 0.5	1.33; 1.58	y;n[b]
5	ND;ND	2.5; 1.5	3.58; 2.58	y;y
6	ND;ND	4.5; >4.0	5.58; >5.08	y;y
7	ND;ND	2.25; 1.75	3.32; 2.83	y;y
8	ND;ND	2.0; 2.0	3.08; 3.08	y;y
9	ND; ND	3.0; 2.25	4.08; 3.33	y;y
10	ND;ND	3.5; 1.5	4.58; 2.58	y;y
11	ND; ND	0.5; 0.5	1.58; 1.58	y;y
12	ND;ND	1.25; 1.25	2.33; 2.33	y;y
13	ND;ND	1.75; 1.0	2.83; 2.08	i[b];i[b]
14	ND;ND	0.5; 2.0	1.58; 3.08	i;y
15	ND;ND	2.5; 3.0	3.58; 4.08	y;y
16	ND;ND	2.5; 1.75	3.58; 2.53	y;y
17	ND;ND	2.0; 2.0	3.08; 3.08	y;y
x	Insufficient data		2.91	
SD			0.93	
RSD%			32.01	
SD_r			0.60	
RSD_r%			20.67	
r			1.69	
SD_R			1.03	
RSD_R%			35.19	
R			2.88	

Table 4

Log_{10} GNB and Log_{10} EU per g of meat from blind duplicate samples of unirradiated skinless chicken breast.

Laboratory	GNB/g	LAL Titre	EU/g	Irradiated (Y/N)
1	5.25; 4.78	1.75; 0.75	1.83; 2.83	n;n
2	5.61; 5.61	3.0; 1.5	4.08; 2.58	n;n
3	5.71; 5.30	1.0; 2.0	2.08; 3.08	n;n
4	4.551; 5.69	0; 2.25	ND[(a)]; 3.33	i[(b,c)];n
5	3.70; 6.41	3.25; 2.0	4.33; 3.08	i[(b)];n
6	5.81; 5.72	3.5; 1.0	4.58; 2.08	n;n
7	6.34; 4.72	3.75; 1.25	3.83; 2.33	n;n
8	5.18; 6.15	4.0; 4.0	5.08; 5.08	n;n
9	3.92; 4.11	2.25; 1.75	3.33; 2.83	n;n
10	5.38; 5.83	1.5; 2.75	2.58; 3.83	n;n
11	5.28; 5.40	1.25; 1.0	2.33; 2.08	n;n
12	5.20; 5.92	1.75; 1.5	2.83; 2.58	n;n
13	5.15; 5.48	3.0; 2.75	4.08; 3.65	n;n
14	5.30; 4.93	0.75; 0.5	1.83; 1.58	n;n
15	6.11; 5.56	1.5; 1.25	2.58; 2.33	n;n
16	4.64; 6.18	2.0; 2.25	3.08; 3.33	n;n
17	4.53; 6.20	1.75; 2.0	2.83; 3.58	n;n
x	5.42		3.07	
SD	0.25		0.78	
RSD%	4.69		25.51	
SD_r	0.77		0.73	
RSD_r%	14.26		23.90	
r	2.17		2.05	
SD_R	0.60		0.94	
RSD_R%	11.12		30.59	
R	2.17		2.63	

Table 5

Log_{10} GNB and Log_{10} EU per g of meat from blind duplicate samples of skinless chicken breast irradiated at 2.5 kGy.

Laboratory	GNB/g	LAL Titre	EU/g	Irradiated (Y/N)
1	ND; <3.48	1.75; 1.5	2.83; 2.58	y;y
2	ND;ND	2.5; 1.0	3.58; 2.08	y;i[b]
3	<3.48; ND	0.25; 1.0	1.33; 2.08	i;y
4	ND;ND	0; 1.25	ND[a]; 2.33	i;y
5	ND;ND	1.5; 1.25	2.58; 2.33	i[b]; i[b]
6	ND;ND	1.5; 1.25	2.58; 2.33	i[b]; i[b]
7	ND;ND	1.0; 2.0	2.08; 3.08	y;y
8	ND;ND	1.5; 2.75	2.58; 3.83	i[b]; y
9	ND; ND	2.5; 2.75	3.58; 3.83	y;y
10	ND;ND	1.75; 1..0	2.83; 2.08	y;y
11	ND; ND	1.75; 1.75	2.83; 2.83	y; i[b]
12	ND;ND	1.25; 1.75	2.33; 2.83	y;y
13	ND;ND	1.75; 1.5	2.83; 2.58	i[b]; i[b]
14	ND;ND	0.75; 0.5	1.83; 1.58	i;i
15	ND;ND	0.5; 1.0	1.58; 2.08	i;y
16	ND;ND	2.0; 1.75	3.08; 2.83	y;y
17	ND;<3.48	1.75; 1.75	2.83; 2.83	y;y
x	Insufficient data	2.60		
SD		0.53		
RSD%		20.27		
SD_r		0.46		
RSD_r%		17.86		
r		1.30		
SD_R		0.62		
RSD_R%		23.88		
R		1.74		

Table 6

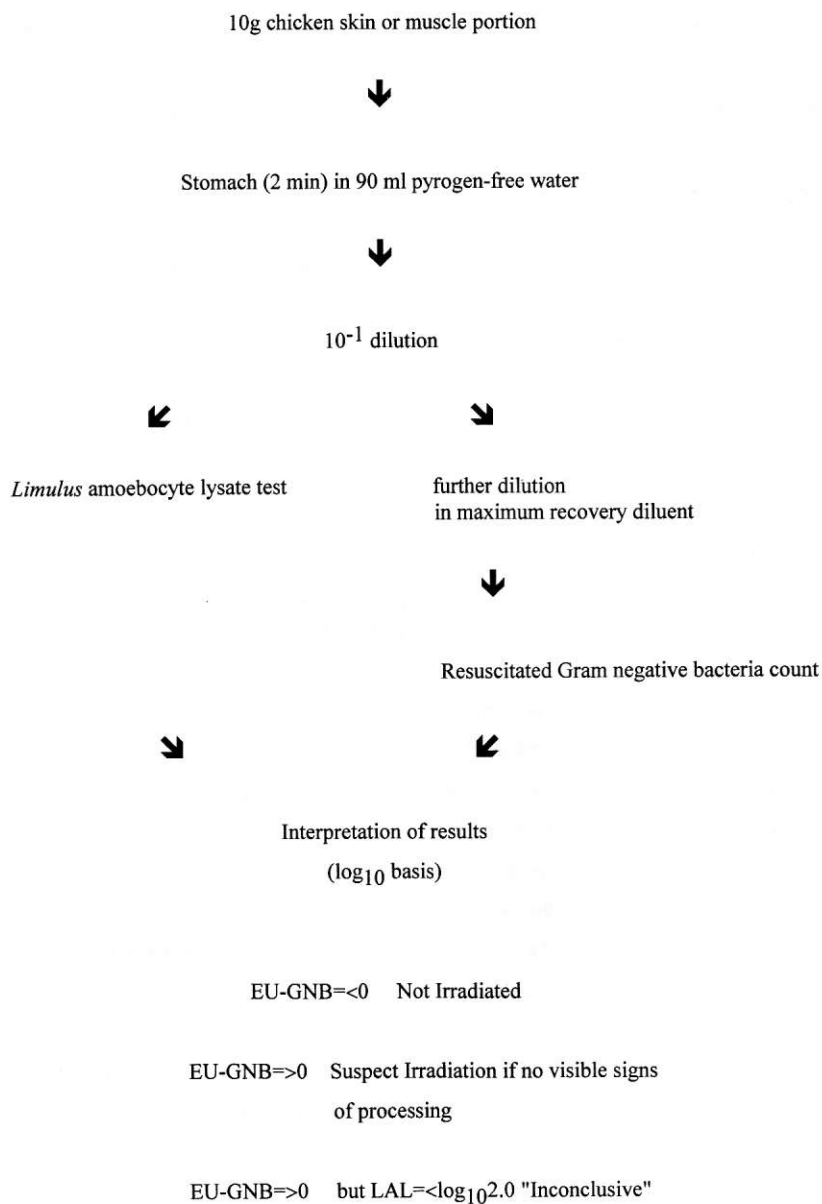
Summary of precision characteristic for LAL test and GNB count (Log_{10})

Sample type	GNB count		LAL test	
	r	R	r	R
unirradiated chicken + skin	1.47	1.82	1.74	2.60
unirradiated chicken - skin	2.17	2.17	2.05	2.63
chicken + skin irradiated at 2.5 kGy	nc	nc	2.12	2.25
chicken + skin irradiated at 5 kGy	nc	nc	1.69	2.88
chicken - skin irradiated at 2.5 kGy	nc	nc	1.30	1.74

Key to Tables 1 - 5

*	: as indicated by participants
ND	: not detected
(a)	: not used in calculations
(b)	: incorrect interpretation by participant
(c)	: LAL plate error
r	repeatability (within laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under the same conditions may be expected to lie with 95% probability.
SD_r	: the standard deviation of the repeatability
RSD_r%	: the relative standard deviation of the repeatability SD_rx100/x
R	: reproducibility (between-lab variation).The value below which the absolute difference between two single test results obtained with the same method on the identical test material under different conditions may be expected to lie with 95% probability.
SD_R	: the standard deviation of the reproducibility.
RSD_R%	: the relative standard deviation of the reproducibility SD_Rx 100/x

Figure 1
Flow diagram of procedure



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Analytical Strategies to Confirm Gin Authenticity

Ross I. Aylott

United Distillers, International Technological Services, Glenochil, Menstrie, Clackmannanshire, UK, FK11 7ES

Analytical methods are required by trading standards and regulatory authorities to confirm the authenticity of gin brands. The characteristics of gin are primarily influenced by the botanical materials used to flavour the product. Analysis of botanical congeners by capillary column gas chromatography showed that specific gin brands had consistent chromatographic profiles over many production batches. Thus their gas chromatographic determination and visual comparison with reference samples offers an effective approach to gin authenticity analysis.

Keywords: gin; authenticity; chromatography

Introduction

Authenticity issues affect a wide variety of products including food and beverages. In an earlier paper we described issues relating to the detection of the on-trade substitution of one brand of Scotch whisky by another, usually a cheaper brand [1]. Similar issues affect other popular spirit drinks including gin.

Gin is based on neutral alcohol, distilled or flavoured with juniper and other botanical materials. Although gin had its origins in Holland (as genevre), it is London Dry Gin which became popular Worldwide and is discussed in this paper. Traditional London Dry Gin is made by distilling neutral alcohol and water in the presence of juniper berry (*Juniperus communis*) and other botanical substances such as coriander seed (*Coriandrum sativum*), angelica root (*Archangelica officinalis*) and orange and lemon peel (*Citrus sinensis aurantium limon*) in a batch process using traditional copper pot stills [2 - 6]. The resulting high strength distillate may simply be reduced with water or a strong flavour distillate may be compounded with neutral alcohol and then reduced with water to give the final distilled gin as a bottled product.

The European Spirit Drinks Regulations define gin as being produced by flavouring of organoleptically acceptable ethyl alcohol of agricultural origin (neutral alcohol) with natural and/or nature identical flavouring substances so that the taste is predominantly of juniper [7]. "Distilled gin" is further defined as having been distilled in traditional pot stills in the presence of juniper and other natural botanicals. This term also applies to mixtures of distilled gin and neutral alcohol. "Compounded gins" are simple mixtures of neutral alcohol, juniper based flavours, essences and water and may not be designated as

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"distilled gin". Similar regulations exist in the USA [8], Canada [9], Australia [10] and many other countries. The minimum alcoholic strength by volume for release for human consumption in the European Union (that is the minimum bottling strength) is 37.5 %, 40% v/v in the USA and Canada and 37 % v/v in Australia. Strengths up to 47 % v/v are often found in products sold in duty-free markets.

The analytical characteristics of gin are influenced by the three principal components making up the product, namely alcohol, water and flavours. Neutral alcohol for gin, vodka and other flavoured spirit manufacture in the European Union must be distilled at a minimum alcoholic strength of 96% v/v. Its characteristics are defined in Annex I of the European Regulations describing ethyl alcohol of agricultural origin [7]. Neutral alcohol is essentially free of congeneric material above trace (< 10 ppm) concentrations [2]. A range of chromatographic and non-chromatographic methods of analysis for checking compliance with the European Regulations have been published by the European Commission [11]. Neutral alcohol for gin manufacture may be fermented from a number of carbohydrate sources including grain (maize or wheat), molasses and lactose. Water for gin distilling and strength reduction is required to be chemically and organoleptically pure. It is normally subject to a demineralisation process before use to render it neutral and low in trace ions [2]. The flavour characteristics of gin are influenced by the nature, quality and quantity of botanical materials used in the distillation. Ensuring the quality of the neutral alcohol and water and careful selection of botanical materials make gin a very stable product [12].

Different gins exhibit a variety of sensory characteristics for which a vocabulary of descriptive terms has been developed [13]. A variety of analytical characteristics relating to the flavour components of different brands may also be expected. Analysis of botanical congeners by capillary column gas chromatography has shown the presence of a wide range of compounds including terpenes, terpineols and sesquiterpenes [2, 14, 15]. As expected, many of these congeners can be associated with the individual botanical materials used such as juniper [16 - 19], coriander [20 - 22] and angelica [23, 24]. This paper demonstrates how the chromatographic fingerprints from such analyses may be used to check the authenticity of specific brands of gin.

Experimental

Apparent alcoholic strength was measured directly with an Anton Paar (Graz, Austria) model DMA 55 precision density meter. pH was determined on various standard instruments. Trace potassium, magnesium and calcium were determined by flame atomic absorption spectroscopy (Unicam 9400, Cambridge, UK) at 766.5, 422.7 and 285.2 nm, respectively, with direct sample aspiration. Sodium was determined by flame atomic emission spectroscopy on the same instrument at 589.0 nm. An acetylene-nitrous oxide flame was used for calcium and an acetylene-air flame was used for sodium, potassium

and magnesium. Calibration standards for each metal were prepared in an aqueous solution of 40% v/v ethanol.

Methanol and other trace volatile congeners were determined by direct-injection (1μl) gas chromatography (Phillips PU4550, Cambridge, UK), with flame ionisation detection, in the presence of pentan-3-ol as internal standard. A 2m × 2mm i.d. glass column packed with 5 % of Carbowax 20M on Carbopak B, 80-120 mesh (Supelco, Bellefonte, PA, USA), was used, temperature programmed from 70° to 160°C at 6°C/min with the injector at 160°C and the detector at 250°C [25]. Sample preparation involved adding pentan-3-ol internal standard solution in 40% ethanol (0.5 ml) to the sample (9 ml) to give a target concentration of 200 μg/g and recording internal standard and sample masses. Congener concentrations were calculated on a mass basis, by means of response factors and peak areas, on a VG (Altricham, UK) Multichrom data system. Congener concentrations were expressed as g per 100 l of absolute alcohol, this being the normal unit in the alcoholic beverage industry.

Botanical congeners were extracted from gin samples (10ml) into n-hexane (1ml) in the presence of ethyl heptanoate as internal standard. Knowing that different gins may be sold at different alcoholic strengths, it was necessary to normalise all gin and reference standards to a fixed alcoholic strength (such as 37.5% v/v) prior to extraction in order to obtain a constant partition of botanical congeners between the gin and extracting solvent. Sample preparation involved adding ethyl heptanoate internal standard in 40% ethanol (0.1 ml) to the gin sample to give a target concentration of 5 μg/g followed by extraction with shaking for 5 min and centrifugation (500g, 0°C, 5 min).

A sample of the extract (1 μl) was analysed by capillary column gas chromatography (Pye Unicam model 204, Cambridge, UK) with on-column injection and flame ionisation detection on a 25 m × 0.2 mm i.d. CP57 CB colunm (Chrompack, Middelburg, Holland). The temperature programme was 40°C for 3 min and 40 to 180°C at 6°C/min. This separation was also examined using vapourising splitless injection as an alternative to on-column injection under similar chromatographic conditions. Congener concentrations were calculated on a mass basis, by means of response factors and peak areas, on a Trivector (Sandy, UK) model 3000 Chromatography Data System.

Chemical standards were obtained from Aldrich (Milwaukee, WI, USA) with the following exceptions: α-humulene (Sigma, St Louis, MO, USA), standard metal solutions (BDH, Poole, UK) and Absolute Alcohol 100 (Hayman, Witham, UK). Sabinene was quantified using the response factor for myrcene as it was not possible to obtain a pure standard.

Results and Discussion

Gas Chromatographic Analyses

The chromatographic procedures described above resulted in two chromatograms. The first, for trace volatile compounds such as methanol and higher alcohols, confirmed the virtual absence of such congeners in the neutral alcohol used in distilled gin manufacture, the only peaks normally detectable being those for ethanol and trace methanol. This observation correlated with earlier published work on the detection of illicit spirits [26].

The second chromatogram for botanical congeners identified a range of terpenes, terpineols, sesquiterpenes and related compounds contributed by the individual flavouring ingredients used. Two examples of this analysis for extracts of brands B and E are shown in Figure 1 with peak identities based on the retention characteristics of reference standards and published work on gin [14]. Ethyl heptanoate was selected as internal standard for this analysis because it was not detectable in gin, it readily extracted into the non-polar solvent and it had a retention time in an area of the chromatogram free of congeners between the terpenes and less volatile terpineols and sesquiterpenes.

Whilst most of the botanical congener analysis reported here was conducted using on-column injection, vapourising splitless injection also proved applicable as in the case of the chromatograms shown in Figure 6. It was also found that similar satisfactory separations could be conducted on other polar capillary columns from various suppliers.

Many monoterpenes such as α- and β-pinene, sabinene, myrcene, and γ- terpinene are contributed by juniper [16 - 19], coriander [20 - 22] and angelica root [23,24]. Citrus materials such as orange and lemon peels contribute the same congeners with proportionately larger amounts of limonene [27, 28]. The contribution of the terpineols and sesquiterpenes are more specific to particular botanical materials. Camphor, linalool and geranyl acetate come mainly from coriander. Terpinen-4-ol, α-humulene and the sesquiterpenes which follow labelled as peaks 14 (γ-muurolene), 16 (δ-cadinene) and 18 in Fig. 1 come mainly from juniper [2, 14].

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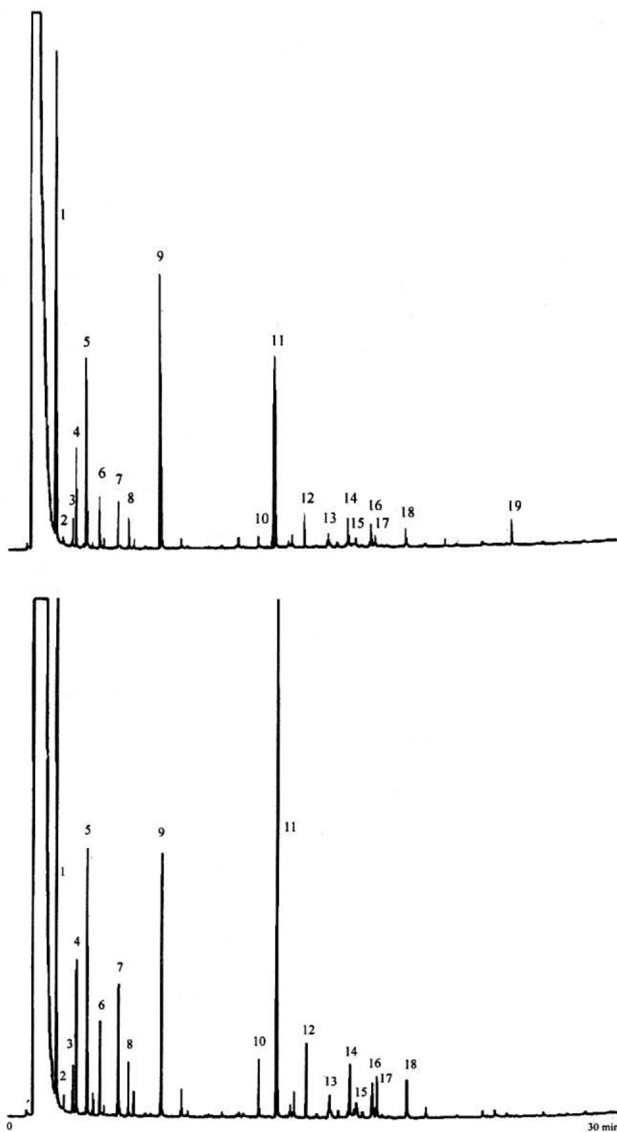
Fig. 1



Capillary column gas chromatograms of extracts of gin brands B (upper) and E (lower): 1, α-pinene; 2, camphene; 3, β-pinene; 4, sabinene*; 5, myrcene; 6, limomene; 7, γ-terpinene; 8, p-cymene; 9, ethyl heptanoate (internal standard); 10, camphor; 11, linalool; 12, terpin-4-ol; 13, α-humulene; 14, sesquiterpine (γ-muurolene*); 15, α-terpineol; 16, sesquiterpine (δ-cadinene*); 17, geranyl acetate; 18, sesquiterpine*; 19, cinnamaldehyde.

* denotes congeners for which reference standards were unavailable.

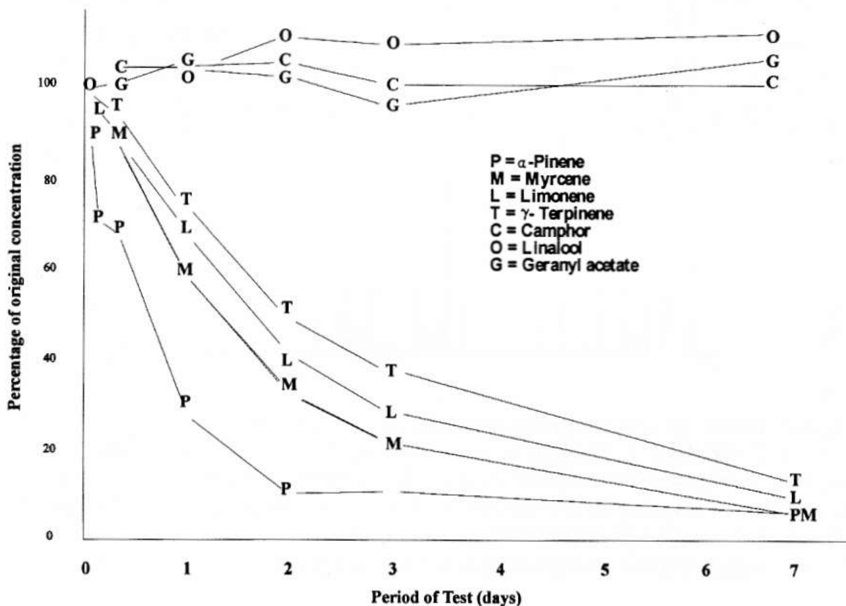
Sample Stability

Whilst it was recognised that gin is a stable product in a closed bottle, it was observed that many volatile terpenes may be lost from gin on prolonged exposure of a sample to the atmosphere. A half filled 70 cl bottle of Brand E was left uncapped for 7 days under ambient laboratory conditions at 20°C. Samples were removed after 0, 2, 4, 8 and 24 hours and 2, 3, and 7 days and their botanical congeners quantified. A similar half filled bottle was capped and examined under similar conditions.

The results indicated losses of volatile monoterpenes from the uncapped bottle while concentrations of the remaining less volatile congeners were much more stable. Fig. 2 shows the percentage of original concentration with time for a range of 7 congeners across the volatility range of the chromatogram. At least 50% of the monoterpenes had been lost within 2 days while the less volatile camphor, linalool and geranyl acetate remained largely unchanged. Losses from the capped bottle, which was only opened to remove samples, were much reduced. α-Pinene lost 25 % after 9 bottle openings while losses of congeners eluting beyond the internal standard were not detectable. From these observations it was concluded that the monoterpenes (eluting before the internal standard) may exhibit considerable concentration variation due to the use history of a sample. The less volatile terpineols and sesquiterpenes (eluting after the internal standard) were much more stable and therefore more relevant to data assessment in authenticity analysis.

Fig.2

Loss of congeners from an uncapped, half filled 70 cl bottle of brand E over a 7 day period under ambient laboratory conditions at 20°C.



Furthermore, the bottles and closures used for collecting suspect samples can influence botanical congener analysis. For example, non-polar terpenes were lost from samples stored in bottles with closures fitted with polyethylene wads. This loss was attributed to adsorption of congeners onto non-polar plastic surfaces. This problem was eliminated by use of inert containers [29]. Our experience is that glass or PET [poly(ethylene terephthalate)] bottles with closures fitted with Melinex (PET film) lined wads are suitably inert.

Congener Analyses

The potential application of these analytical techniques to confirm the authenticity of specific gins was examined by using analytical data generated over a three year period from seven different brands labelled A - G. Individual new bottles of each brand were purchased on a quarterly basis and subjected to the full analysis described under Experimental. Acquisition of the samples on a quarterly basis increased the probability that each sample was produced from a different production batch and gave a total of 12 samples for each brand (the exceptions being 7 samples of brand B and 10 samples for brand C). Data collected included alcoholic strength, pH, higher alcohols and botanical congeners.

Examination of the results for the 7 brands showed that alcoholic strength always fell within 0.1% v/v of that declared on product labels. pH values were generally neutral and within the range 5 to 8.5. By reference to the pH of demineralised water used for in-house brands, it could be seen that the pH of the gins reflected the pH of the demineralised water used to reduce their high strength gin distillates to bottling strength. Concentrations of trace metals were all low, again reflecting the demineralised water used for strength reduction. For example, concentrations were <0.1 μg/ml for all 4 metals monitored in at least 90% of samples, with occasional excursions up to 5, 2 and 2 μg/ml for sodium, potassium and calcium, respectively.

These results for normal samples of distilled gins provide the analyst with useful background data when examining suspect samples with alcoholic strengths considerably lower than that declared on the product label, with abnormal pH values and with high trace metal concentrations. Such suspect samples may have been contaminated with mixer drinks such as soda and tonic waters. For example, a suspect gin sample containing traces of soda water may have low alcoholic strength and a high sodium concentration, one containing tonic water may contain quinine and lime oil and other mixer drink combinations may give rise to the trace presence of benzoate preservatives and citric and phosphoric acids which reduce pH.

Gas chromatographic higher alcohol analysis indicated that all the gins were based on neutral alcohols which were generally free of congeneric material. The only trace congener regularly detected was methanol at concentrations up to 4 g per 100 1 absolute alcohol, other congeners potentially detectable in this chromatogram, such as

acetaldehyde, ethyl acetate, isobutanol and isoamyl alcohols, were not detected (with limits of detection of <0.5 g per 100 1 absolute alcohol). *n*-Propanol was detected on 3 occasions at concentrations up to 6.5 g per 100 l absolute alcohol. In summary, alcoholic strength, pH and higher alcohol results were similar for all the brands examined and, while demonstrating characteristics common for a range of gins, did not form the basis of brand authenticity analyses.

Examination of the botanical congener chromatograms for these brands showed that the same congeners were present in most of the brands but at considerably differing concentrations. The two exceptions were brands B and C which contained trace cinnamaldehyde in addition to the congeners present in the other five brands. Botanical concentration differences between the brands were evident when examining quantitative data as shown in Table 1 for α-pinene, sabinene, myrcene, limonene, γ-terpinene, camphor, linalool and terpinen-4-ol. These results showed that concentrations of α-pinene ranged from 0.7 to 46 g per 100 1 absolute alcohol in all the brands examined but were also relatively variable within individual brands, reflecting the volatility losses described above. The analytical ranges for sabinene, myrcene and γ–terpinene also showed considerable overlap, but with brands B and C having concentrations at the lower ends of the ranges.

Table 1

Analytical Ranges and Averages (in parentheses) for Samples of Each of Seven Gin Brands Labelled A-G

	Brand A	Brand B	Brand C	Brand D	Brand E	Brand F	Brand G
Number of samples	12	7	10	12	12	12	12
α-Pinene	5.3 - 46 (24)	6.7 - 16 (12)	0.7 - 9 (5.2)	5 - 24 (15)	3 - 32 (19)	6.1 - 38 (23)	2.8 - 17 (7.1)
Sabinene	1.6 - 8.2 (5.2)	1.4 - 2.7 (2.1)	0.5 - 1.7 (1.2)	3.0 - 6.4 (4.9)	1.8 - 8.0 (4.8)	2.2 -7.1 (4.3)	1.5 - 5.5 (3.0)
Myrcene	3.6 - 9.1 (7.2)	3.3 - 4.2 (3.9)	1.0 - 2.6 (2.1)	4.0 - 8.6 (6.9)	3.6 - 9.1 (7.0)	3.6 - 7.4 (5.8)	2.2 - 6.8 (4.1)
Limonene	11-26 (20)	1.0 - 1.4 (1.2)	0.4 - 0.9 (0.7)	15 - 32 (24)	2.1 - 3.8 (2.8)	1.5 - 2.9 (2.2)	1.8 - 3.7 (3.0)
γ-Terpinene	2 - 5 (4)	1.0 - 1.4 (1.2)	0.4 - 0.8 (0.6)	3.9 - 6.8 (5.1)	2.3 - 4.4 (3.2)	1.8 - 3.1 (2.5)	2.8 - 4.4 (3.7)
Camphor	0.8 - 1.8 (1.2)	0.3 - 0.4 (0.4)	0.1 - 0.2 (0.2)	1.6 - 2.7 (2.0)	1.3 - 3.2 (2.0)	0.6 - 1.7 (1.1)	1.9 - 2.9 (2.4)
Linanool	14 - 26 (19)	1.6 - 8 (5.4)	2.3 - 3.2 (2.7)	28 - 43 (35)	19 - 41 (29)	10 - 27 (17)	32 - 44 (37)
Terpinen-4-ol	2.0 - 6.7 (3.9)	0.8 - 1.4 (1.0)	0.4 - 0.7 (0.5)	1.5 - 5.0 (3.3)	2.1 - 4.7 (3.2)	0.9 - 3..3 (1.9)	1.5 - 2.8 (2.3)
Cinnamaldehyde	ND (ND)	1.2 - 2.4 (1.8)	0.6 - 1.3 (0.8)	ND (ND)	ND (ND)	ND (ND)	ND (ND)

Concentration units: g per 100 l absolute alcohol ND = Not Detected

Limonene concentrations for brands A and D were distinctly higher that the others, presumably due to the relatively high proportions of citrus based botanical materials used in their distillation (Fig. 3). Linalool concentrations fell into three clusters encompassing brands B and C at the lower end; brands A and F in the middle; and brands D and G at the upper end. Brand E fell within the middle and upper clusters (Fig. 4). Camphor

Fig.3

Limonene concentrations in 12 samples of each of 7 gin brands labelled A-G

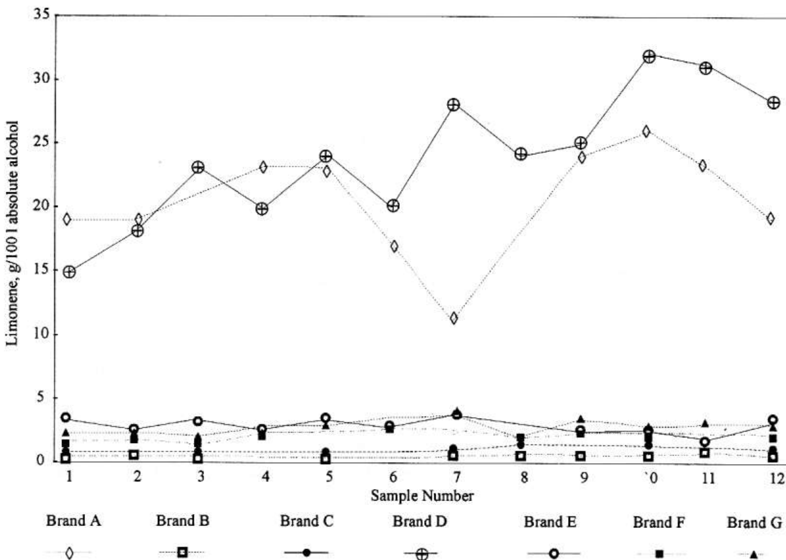
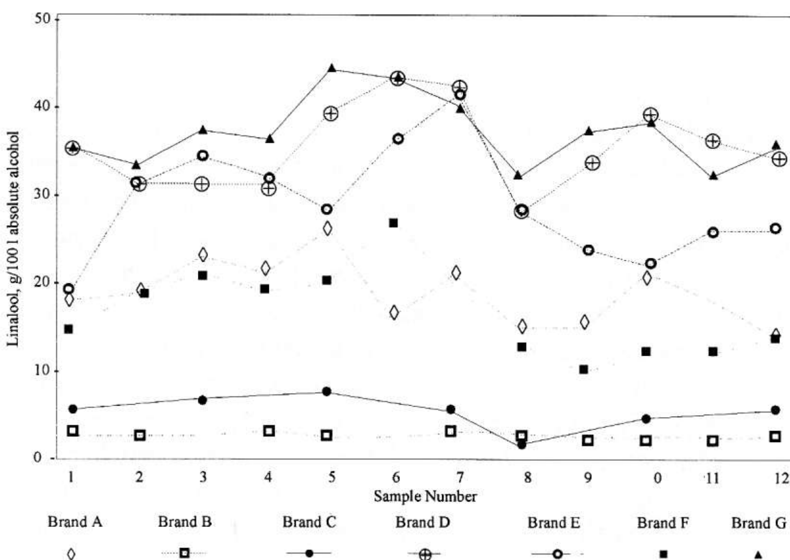


Fig.4

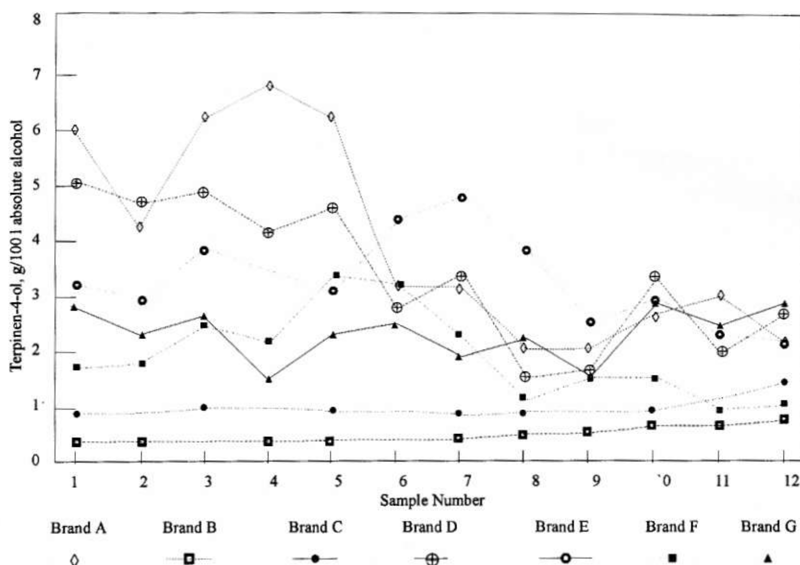
Linalool concentrations in 12 samples of each of 7 gin brands labelled A-G



results reflected those for linalool as both these congeners originate from the same botanical material [20 23]. Terpinen-4-ol results for brand B and C formed a low cluster while the results for the remaining brands overlapped (Fig. 5).

Fig.5

Terpinen-4-ol concentrations in 12 samples of each of 7 gin brands labelled A-G



Applications

Applications of this information in developing a strategy for brand authenticity checking were demonstrated using data from 3 suspect samples purporting to brand E as described in Table 2. Suspect Sample 1 had a low alcoholic strength (37.35% compared to 40% v/v declared on the label). The concentrations of the less volatile botanical congeners, except α-pinene and limonene, were below the normal ranges for brand E but these results were not considered particularly significant knowing that these congeners are readily lost from the product by evaporation. More interestingly the concentration of the less volatile congenersterpinen-4-ol was below it's normal range and raised doubt on sample authenticity. Inspection of the botanical congener capillary chromatogram for Sample 1 against a genuine reference sample of brand E (Fig. 6) demonstrated these concentration differences and the different relative proportions of certain congeners. For example, suspect Sample 1 contained a higher concentration of limonene (peak 6 in Fig. 6) relative to its neighbouring peaks while limonene was relatively low in the reference

sample. Finally, the higher alcohol chromatogram showed the presence of isopropanol, a component not normally detected in distilled gins. The presence of isopropanol suggested that sample 1 was a compounded gin produced from flavours in which isopropanol acted as solvent/carrier. The combination of low strength, low congener concentrations and the presence of isopropanol led to the conclusion that Sample 1 was not authentic brand E.

Table 2

Results for suspect samples 1-3 (for comparison with brand E in Table 1)

	Sample 1	Sample 2	Sample 3
Alcoholic Strength, % v/v at 20°C			
- Label strength	40	37.5	40
-Apparent strength	37.35	37.4	40.17
Botanical congeners, g/100 l absolute alcohol			
α-Pinene	3.8	5.2	24
Sabinene	0.1	0.2	5.1
Myrcene	0.5	2.6	7.5
Limonene	2.9	4.1	3.3
γ-Terpinene	1.1	2.1	4.5
Camphor	1.9	0.5	3.0
Linalool	24	3.9	39
Terpinen-4-ol	0.4	4.3	2.0
Cinnamaldehyde	ND	ND	ND
Other components	isopropanol	none	none

ND = Not Detected

Suspect sample 2 had concentrations of most of its volatile congeners near or below the lower end of the normal range for brand E, but the concentration of limonene was above the range and relatively high compared to adjacently eluting congeners. The concentrations of the less volatile camphor and linalool were below brand E ranges while terpinen-4-ol was within range. The atypically high limonene and low camphor and linalool concentrations led to the conclusion that sample 2 was not authentic brand E.

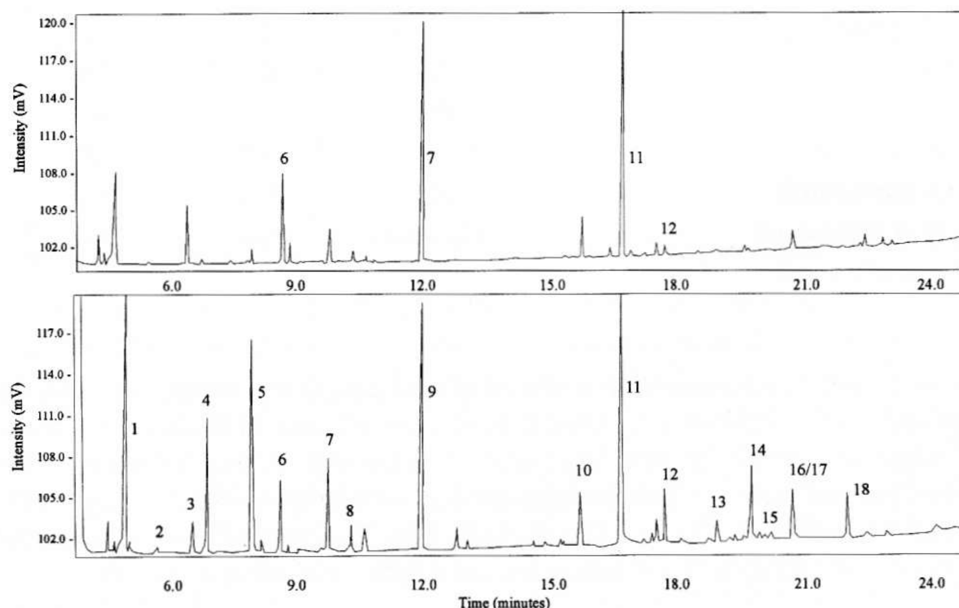
Suspect sample 3 had congener concentrations within range for brand E, with the exception of γ-terpinene which was slightly above the top of the range and terpinen-4-ol which was slightly below the lower end of the range. As these differences were not analytically significant it was concluded that sample 3 was consistent with brand E.

Conclusions

Botanical congener analysis by capillary column gas chromatography provided a valuable method for checking gin brand authenticity with particular attention being paid to the concentrations of the less volatile congeners eluting in the chromatogram between camphor and cinnamaldehyde. Visual comparison of the chromatographic profiles of suspect and reference samples is appropriate, again with most attention being paid to the less volatile congeners. In addition, abnormal alcoholic strengths and the presence of foreign peaks in the higher alcohol and botanical congener chromatograms may give the analyst useful information. The potential exists for this analytical strategy to be further developed by examining other congeners in the botanical congener chromatogram, calculating various peak ratios and by applying chemometric techniques in the assessment of the chromatographic data.

Fig. 6

Capillary column gas chromatograms of extracts of suspect gin sample 1 (upper) compared to a reference sample of gin brand E (lower) using vaporising splitless injection and a VG Multichrom data system. Peak designation is given in Figure 1.



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