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A STUDY OF THE EFFECTS OF INSTRUMENT VARIABLES ON ACCURACY AND PRECISION IN GAS CHROMATOGRAPHY

II. ERRORS ASSOCIATED WITH THE INJECTION OF LIQUID SAMPLES BY MICROLITRE SYRINGES

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SUMMARY

Statistically designed experiments have been carried out to examine the effects of operator technique and injection variables on the precision and accuracy of peak height and area ratios. The results show that technique must be carefully standardized and that bias errors can be minimized by sound chromatographic practice. In general, on-column injection gave significantly better precision for narrow-boiling-range mixtures, but flash vaporization injection gave better results for peak height ratios with wide-boiling-range mixtures.

INTRODUCTION

This paper is the second in the series to be prepared from the results of the BSI Research Project described in Part I (ref. 1). The purpose of this study was to assess the nature and magnitudes of errors that occur in liquid sample introduction to packed columns using microlitre syringes. This operation probably contributes a larger total error to the overall analytical error than any other single source. The total error arising from sample introduction can consist of random error, which leads to lack of precision, and bias error, which leads to lack of accuracy.

Nevertheless, the injection of liquid samples into the chromatograph by means of a microlitre syringe is now the accepted method for the majority of applications and this situation is unlikely to change in the forseeable future. Thus, whatever the inherent disadvantages of the method, the principal aim of this study was to examine the errors associated with the normal procedure rather than to attempt to devise more precise and accurate methods of sample introduction.

Clearly there are two major sources of error arising from the injection process, *viz.*, procedural error, which is caused by the operator and the particular technique he employs for filling the syringe and discharging it in the injection system, and instrumental error, caused by the design of the injection system and the conditions

under which it is operated. A complete factorial investigation of all the possible variables would have been prohibited by the limited time available and so these two major sources of error were investigated separately because the probable interactions can be predicted by simple logic.

NATURE OF INJECTION ERRORS

The ideal method of syringe injection would add the required volume of a representative fraction of the sample to the stationary phase in the first theoretical plate. This could be achieved, in principle, by displacing such a fraction from the syringe directly on to the column packing by the depression of the plunger. In practice, however, the syringe needle invariably contains residual sample and fractionation effects will occur while the syringe needle is immersed in the injection system. This can lead to a relative enrichment of the lower-boiling components, the extent of which depends on both the residence time and the temperature of the system. When relatively low vaporizer temperatures are used, as is often the case with on-column injection, it is sound practice to withdraw the syringe immediately after the depression of the plunger to minimize this fractionation effect. When high vaporizer temperatures are used, however, as is often the case in flash vaporization, significant fractionation occurs concurrently with sample displacement and so it may be expedient to use a longer residence time to vaporize all the residual sample from the needle. This technique, of course, would only be practised with very small capacity syringes, where the needle acts as the barrel and the sample is displaced by the movement of a steel wire inside the needle.

With syringes of larger volume, the needle is always completely filled with sample and serious bias errors due to fractionation would occur if the needle were not removed immediately after the depression of the plunger. In practice the injection technique usually consists of a combination of flash vaporization and on-column injection and the optimum residence time can only be estimated by trial runs on typical samples. Thus, we see that by the application of sound chromatographic principles, bias errors that arise from fractionation effects should be minimized. However, there is a further problem, which arises from a combination of operator technique and the injection conditions. This is the effect of injection variables on the "injection function", *i.e.*, the distribution of the solute zone on entering the column packing. This can be adversely affected by capacity effects in the system, overloading the stationary phase and by slow vaporization of components. The use of low vaporizer temperatures w tend to give a poor injection function for high-boiling components causing tail g peaks. Conversely fractionation effects from the needle will have a more critical effect on the zone widths and hence the heights of rapidly eluted compounds than on those of well-retained compounds. With either of these effects it is good practice to use peak area rather than peak height measurements, but again the appropriate procedure can only be chosen from the results of trial runs.

EXPERIMENTAL

For the purposes of the investigation the errors arising from syringe injection were considered to be of two main types, *viz.* (i) Operator errors resulting from the

technique adopted for filling the syringe and injecting the sample. This is defined as "procedural error". (ii) Errors resulting from the design of the injection system and its operating conditions. This is defined as "instrumental error".

The microsyringe may also affect both of these types of error from the viewpoint of both its design and degree of wear. In this investigation it would have been inappropriate to compare syringes of different origin, but experience has shown that all microsyringes deteriorate gradually with use. Thus, any excessive error resulting from the use of a particular syringe was included in the replicate error by using several syringes during the investigation. In view of the limited time available for this study only 1-μl "plunger in needle" syringes were employed. The injection system consisted of a separately heated block or "vaporizer" that could be adapted to either flash vaporization or on-column injection. In either case the syringe needle penetrates a silicone rubber septum and projects into the centre of the block. For flash vaporization injection this central space is occupied by a 10-cm stainless-steel tube that is connected to the column inlet outside the block. For on-column injection the column inlet itself is inserted into the block so that the syringe needle can make contact with the packing. The inlet gas stream enters the column around an annular space between the outside of the stainless-steel tube (or the column) and the wall of the vaporizer. This annular space forms a constriction to minimize the possibility of back-diffusion effects.

PROCEDURAL ERROR

A sample was prepared comprising 1 "$_v$ toluene and 1 "$_v$ *cis*-dekalin in cyclohexane. These materials were chosen because of the wide difference in their boiling points, to detect fractionation effects, and because of their thermal stability.

The following chromatographic conditions were used. Chromatograph: Varian 1860-1, fitted with improved flow control system incorporating thermostatted capillary constrictions for the carrier gas and auxiliary gases, and a precision pressure controller situated up-stream from the chromatograph inlet points. Carrier gas: nitrogen at 12 p.s.i. Column: 3 m × 6 mm O.D. stainless steel, packed with 20 "$_w$ Carbowax 20M on Celite, 72–80 BS mesh. Column temperature: 160 °. Detector: flame ionization. Detector oven temperature: 200 °. Recorder: Servoscribe, Type RE 512-20, operated on 10-mV range. Electronic integrator: Varian Aerograph, Model 480. Injection system: flash vaporization mode, temperature 200 °.

A standardized injection procedure was adopted and the effects of deviations from this procedure were studied by carrying out runs in pairs, one sample being injected by the standardized procedure and the other by a variant procedure in which only one variable was altered. The order of the injections was randomized to avoid any possible order effects and sets of eight pairs were run for each variant–standard combination. The peak areas of the toluene and *cis*-dekalin were measured by the electronic integrator and their heights were measured manually in the normal way. Details of the standard and variant procedures and associated set numbers are given in Table I. Sets 4 and 18 were performed without a variant to ensure the absence of any injection order effect or "presence of variant" effect that could cause the operator to be significantly less precise in his sequence of operations when variants are included in the set.

TABLE I

DETAILS OF STANDARD AND VARIANT PROCEDURES

Standard	*Set No.*	*Variant*
Sample taken through "suba-seal" stopper	5	Sample taken through "suba-seal" stopper and syringe wiped carefully, avoiding tip
	6	Sample taken through "suba-seal" stopper and syringe wiped without avoiding tip
	1	Sample taken from open bottle and syringe not wiped
Syringe not wiped	2	Sample taken from open bottle and syringe wiped carefully, avoiding tip
	3	Sample taken from open bottle and syringe wiped without avoiding tip
Plunger raised 3 mm beyond "full" position when filling syringe and then returned to "full" position	17	Plunger not raised beyond "full" position
Syringe filled after three operations of the plunger	7	Syringe filled after one operation of plunger
	8	Syringe filled after ten operations of plunger
Immediate injection	16	20-sec interval between filling the syringe and injection
No spacer bar used, *i.e.* 52 mm of needle enters the injection system	9	Short spacer bar used. 30 mm of needle enters the injection system
	10	Long spacer bar used. 16 mm of needle enters the injection system
Needle inserted centrally into septum and injection system	15	Needle inserted off-centre and to one side of injection system
Needle inserted rapidly into septum	12	Needle inserted slowly into septum
Plunger depressed rapidly	11	Plunger depressed slowly
Needle left in injection system for 10 sec after complete depression of plunger	13	Needle left in injection system for 2 sec after depression of plunger
	14	Needle left in injection system for 20 sec after depression of plunger
As above	4	No variation
	18	No variation

RESULTS OF INJECTION PROCEDURE TESTS

Table II gives the results for the mean set ratios of toluene to *cis*-dekalin peal areas and heights, with their coefficients of variation, and also the mean set ratios o variant to standard peak ratios with their standard errors.

The mean set area ratios of toluene to *cis*-dekalin decreased gradually durin the period of the experiment owing to volatilization losses. This accounts for the muc greater set variances in sets 10, 16 and 18, which were carried out with night or weel end intervals.

Comparing the coefficients of variation within each set, only sets 5 and 11 i

TABLE II

RESULTS OF PROCEDURAL ERROR INVESTIGATION

Set No.	Ratio (toluene/dekalin) Mean for sets		Coeff. var. (%)		Sig. level of diff. in coeff. var. (%)	Ratio (toluene/dekalin (var) / toluene/dekalin (std)) of pairs		
	Std.	Var.	Std.	Var.		Mean for sets	Standard errors of mean	Sig. diff. from unity (%)
Areas								
1	1.1605	1.1624	0.804	1.05	ns	1.0019	0.00548	ns
2	1.1315	1.1229	0.870	0.859	ns	0.9924	0.00266	99
3	1.1156	1.1123	0.483	0.360	ns	0.9968	0.00141	95
4	1.0816	1.0886	0.413	0.752	ns	1.0067	0.00412	ns
5	1.0790	1.0074	0.278	0.682	95	0.9986	0.00202	ns
6	1.0676	1.0670	0.546	0.701	ns	0.9993	0.00197	ns
7	1.0674	1.0635	0.229	0.364	ns	0.9966	0.00158	95
8	1.0612	1.0671	0.535	0.529	ns	1.0055	0.00235	95
9	1.0612	1.0660	0.263	0.494	ns	1.0045	0.00230	95
10	1.0312	1.0354	2.92	3.08	ns	1.0041	0.00335	ns
11	1.0022	1.0032	0.322	0.890	95	1.0010	0.00326	ns
12	1.0057	1.0013	0.867	1.05	ns	0.9957	0.00569	ns
13	1.0106	1.0128	0.290	0.353	ns	1.0021	0.00207	ns
14	1.0111	1.0105	0.476	0.343	ns	0.9994	0.00155	ns
15	0.9890	0.9846	0.690	0.664	ns	0.9956	0.00434	ns
16	0.9911	0.9841	1.17	1.06	ns	0.9930	0.00581	ns
17	0.9881	0.9832	0.704	0.910	ns	0.9950	0.00192	95
18	0.9583	0.9566	3.08	3.30	ns	0.9982	0.00308	ns
	Overall mean		0.796	0.968	99			
Heights								
1	1.499	1.488	0.615	2.60	99	0.9925	0.00972	ns
2	1.453	1.452	0.788	0.957	ns	0.9993	0.00228	ns
3	1.437	1.431	0.472	0.342	ns	0.9957	0.00110	99
4	1.395	1.399	0.537	0.357	ns	1.0029	0.00226	ns
5	1.393	1.396	0.269	0.320	ns	1.0019	0.00122	ns
6	1.378	1.381	0.775	0.728	ns	1.0021	0.00224	ns
7	1.374	1.372	0.262	0.326	ns	0.9992	0.00190	ns
8	1.369	1.359	0.579	0.684	ns	0.9931	0.00274	95
9	1.367	1.348	0.411	0.623	ns	0.9861	0.00138	99
10	1.328	1.298	3.07	2.35	ns	0.9774	0.00522	99
11	1.292	1.287	0.251	0.482	ns	0.9963	0.00176	95
12	1.291	1.271	0.605	0.618	ns	0.9847	0.00239	99
13	1.291	1.301	0.578	0.277	ns	1.0077	0.00207	99
14	1.303	1.301	0.322	0.457	ns	0.9984	0.00214	ns
15	1.272	1.275	0.371	0.438	ns	1.0027	0.00152	ns
16	1.279	1.282	0.647	0.698	ns	1.0027	0.00375	ns
17	1.276	1.283	1.08	2.90	99	1.0052	0.00990	ns
18	1.232	1.235	3.46	3.82	ns	1.0021	0.00161	ns
	Overall mean		0.838	1.054	99			

the area ratio results give a significantly lower precision using the variant. The comparatively lower precision for set 5 is probably a result of variable fractionation effects from near the needle tip due to it not being wiped. In this event one would also expect significantly worse precisions for variant sets 1 and 2, but here unfortunately much of

the significance is lost owing to the much larger variances arising from the day-to-day volatilization losses. However, the slow depression of the plunger in set 11 clearly has an adverse effect and so should be avoided.

The overall mean set variance for standards is significantly better than for the variants. This confirms that a standardized procedure will give a better precision in injection than a variable one. The general lack of significant differences, however, between the variances of the standard procedure and those of the variants implies that the actual procedure adopted as a standard is not critical, provided that it complies with sound chromatographic practice.

For peak height ratios only sets 1 and 17 gave a significantly lower precision using the variant procedure, and since neither of these sets gave significant effects for the area ratios, the lower precisions experienced in the variant mode must be attributed to injection function effects. Thus, not wiping the needle when the sample is taken from the open bottle or not raising the plunger beyond the full position when filling the syringe will leave sample at the very tip of the needle. This seems to cause some significant peak spreading that affects the toluene peak more critically than the *cis*-dekalin peak.

The results for the variant to standard ratios were calculated to eliminate the time effect caused by preferential losses of toluene and changes in response. When these mean ratios are statistically different from unity, we can assume the existence of bias errors, the nature of which depends on whether the effects were high or low

TABLE III

SUMMARY OF SIGNIFICANT EFFECTS IN VARIANT/STANDARD PEAK AREA AND HEIGHTS RATIOS

*Ratio**		*Application to set numbers*	*Probable explanation*
Areas	*Heights*		
Low	No sig. effect	2, 7, 17	(a) Preferential loss of toluene in variant probably taking place by fractionation from syringe before injection. (b) Preferential loss of dekalin in standard probably due to fractionation after injection in which a higher concentration of dekalin is left behind.
Low	Low	3	(c) As in (a) or (b) together with additional injection function effect due to zone spreading.
High	Low	8, 9	(d) Preferential loss of dekalin in the variant due to fractionation after injection, leaving residual dekalin, with additional injection function effect.
No sig. effect	Low	10, 11, 12	(e) Injection function effect with variant but probably no bias error due to fractionation.
No sig. effect	High	13	(f) Injection function effect with standard but probably no bias error due to fractionation.

* $\text{Ratio} = \dfrac{\text{variant ratio}\ \dfrac{\text{toluene}}{\text{dekalin}}}{\text{standard ratio}\ \dfrac{\text{toluene}}{\text{dekalin}}}$

and whether they were obtained for areas, heights or both. Table III gives a summary of the significant results and their probable explanations.

Regarding the area results, it is apparent that the number of insignificant differences by far exceeds the number of significant differences. This strongly suggests that the standard procedure does, on average, provide a representative sample with no bias error. Thus, in those sets where significant differences occur, the effects are probably due to bias errors in the variant procedure. For the height results the preponderance of low values indicates that the variant procedure is giving greater spread of the toluene peak than the standard procedure and this is because of an injection function effect. Only set 13 gives a high result and it is interesting to compare the height ratio results for sets 13 and 14. There is apparently a decreasing effect with increasing residence time, which suggests that the standard procedure is having a slightly adverse effect on the toluene spread and a shorter residence time would be preferable. This would also reduce the possibility of fractionation effects that could give significant bias errors with lower vaporizer temperatures.

INSTRUMENTAL ERROR

Factorial experiments were designed to test which of the instrumental injection variables cause systematic changes in relative peak areas and heights. The experiments were intended to test the effects of vaporizer temperature, mode of injection, chemical type and boiling range on the representativity of the chromatographed sample, as measured by changes in relative peak areas, and the injection function error, as measured by changes in relative peak heights. Four boiling ranges were selected, each containing components of differing chemical types. Details of the mixtures are given in Table IV.

The chromatographic runs were carried out in sets, each set consisting of short-term duplicates run by the standard injection procedure. Three replicates were performed under each combination of conditions, but on different days and using two different syringes so that errors from these sources were included in the replication error. Experimental details are given in Table V. Each figure refers to a complete set and designates the order of performing the set.

Analyses of variance were performed on the results and the effects were tested for significance using the "F" test. "Within" effects were tested by comparison of estimated precisions with each other and "between" effects were tested by comparison with the estimated mean replicate variance for the particular compound concerned. The results are summarized in Tables VI–IX, which give the estimated percentage relative standard deviations for each effect and the significance of the values obtained.

CONCLUSIONS TO EXPERIMENTS ON INSTRUMENTAL EFFECTS

Within effects

Replicates

Area ratios. With the low-boiling mixture the mean coefficient of variation for area ratio replicates is least for toluene and greatest for 1-propanol, these two values differing by a factor of about ten. The methanol value is also high, suggesting that the

TABLE IV

DETAILS OF MIXTURES USED IN EXPERIMENTS ON INSTRUMENTAL ERROR

r = Reference compound.

Sample type (column temperature)	*Component*	*B.p. (°C)*	*Relative retention (reference = 1.00)*
Low boiling (100°)	Diethyl ether	35	0.31
	Cyclohexane	81	0.43
	Methanol	64.5	0.69
	Benzene (r)	81	1.00
	1-Propanol	97	1.30
	Toluene	111	1.61
Medium boiling (100°)	1-Propanol	97	0.53
	n-Butyl acetate	126	0.69
	Ethylbenzene (r)	136	1.00
	o-Xylene	144	1.36
	cis-Dekalin	195	1.81
High boiling (160°)	Ethylbenzene (r)	136	1.00
	Tetradecane	252	2.11
	Indene	183	3.70
	Hexadecane	287.5	4.63
Wide range (160°)	Diethyl ether	35	0.23
	Cyclohexane	81	0.27
	Toluene	111	0.50
	Ethylbenzene	136	0.64
	cis-Dekalin (r)	195	1.00
	Tetradecane	252	1.32
	Indene	183	2.27
	Hexadecane	287.5	2.80

TABLE V

EFFECT OF INSTRUMENT VARIABLES ON INJECTION — EXPERIMENTAL DETAILS

Type of mixture	*On-column*			*Flash vaporization*			
	Vaporizer temparature (°C)			*Vaporizer temparature (°C)*			
	100	*150*	*200*	*100*	*150*	*200*	*250*
Low boiling	9	5	1	17	21	25	13
	9A	5A	1A	17A	21A	25A	13A
	1B	5B	9B	13B	25B	21B	17B
Medium boiling	10	6	2	18	22	26	14
	10A	6A	2A	18A	22A	26A	14A
	2B	6B	10B	14B	26B	22B	18B
High boiling	12	8	4	20	24	28	16
	12A	8A	4A	20A	24A	28A	16A
	4B	8B	12B	16B	28B	24B	20B
Wide range	11	7	3	19	23	27	15
	11A	7A	3A	19A	23A	27A	15A
	3B	7B	11B	15B	27B	23B	19B

TABLE VI

SUMMARY OF RESULTS OF ANALYSIS OF VARIANCE FOR LOW-BOILING MIXTURE

Reference peak = benzene (peak 4).

Compounds in order of elution	*Percentage relative standard deviations*									
	Within effects						*Between effects*			
	Reps.	*On-column (oc)*	*Flash v.*	*Set 1*	*Set 2*	*Set 3*	*Temperatures*		*Sets*	*Modes*
							oc	*Flash v.*		
	Area ratios									
Diethyl ether	1.41	1.44	1.53	0.75	0.52	0.37	0.45	1.11	3.73, (99)	1.17
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.06, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 4, (90)						
Cyclohexane	1.46	1.20	1.78	0.68	0.65	0.21	1.23	1.23	3.88, (99)	0.65
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.48, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 11, (99)						
Methanol (benzene)	2.85	2.14	3.45	1.38	1.15	0.93	0.78	1.33	7.30, (95)	1.50
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.61, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 2.22, (ns)						
1-Propanol	3.79	2.93	4.49	1.73	1.28	0.89	0.32	1.55	9.79, (95)	1.99
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.53, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 3.8, (90)						
Toluene	0.39	0.16	0.41	0.41	0.21	0.16	0.09	0.27	0.53	0.54
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 7.0, (95)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 7, (95)						
Overall mean	1.98	1.57	2.35	0.99	0.95	0.51	0.58	1.10	—	1.17
d.o.f.*	70	40	55	30	30	30	10	15	—	5
	Height ratios									
Diethyl ether	4.46	6.39	3.26	5.24	5.89	3.53	5.98	8.39, (90)	8.82, (90)	8.07
	$F\left(\frac{\text{oc}}{\text{flash}}\right)$ = 3.83, (90)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 2.2, (ns)						
Cyclohexane	2.35	1.98	3.33	2.06	2.45	1.97	3.28	3.48, (95)	4.10, (95)	1.05
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 2.81, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 1.1, (ns)						
Methanol	2.70	1.82	3.44	1.36	1.84	3.17	2.77	4.53	6.76, (95)	6.20, (95)
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 3.58, (95)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right)$ = 5.47, (95)						
1-Propanol	4.72	3.56	5.80	2.84	1.44	1.08	1.27	1.97	12.30, (99)	2.49
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 2.66, (90)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 6.9, (95)						
Toluene	0.45	0.55	3.38	0.32	0.69	0.95	0.92	2.78, (99)	0.17	3.20, (99)
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 38, (99.9)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right)$ = 9, (99.9)						
Overall mean	2.94	2.86	3.84	2.36	3.08	2.14	3.24	4.23,	—	4.20
d.o.f.*	70	40	55	30	30	30	10	15	—	5

* Degrees of freedom.

TABLE VII

SUMMARY OF RESULTS OF ANALYSIS OF VARIANCE FOR MEDIUM-BOILING MIXTURE
Reference peak = ethyl benzene (peak 3).

Compounds in order of elution	*Percentage relative standard deviations*									
	Within effects						*Between effects*			
	Reps.	*On-column (oc)*	*Flash v. oc*	*Set 1*	*Set 2*	*Set 3*	*Temperatures*		*Sets*	*Modes*
							oc	*Flash v.*		
	Area ratios									
1-Propanol	6.55	4.12	7.05	7.16	2.12	2.70	1.40	5.24	13.1, (95)	3.19
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 3.01, (95)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 5.8, (95)						
Butyl acetate (ethylbenzene)	2.25	1.63	2.38	1.62	0.72	1.47	1.31	1.72	5.35, (95)	2.14
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 2.19, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 1.12, (ns)						
o-Xylene	0.59	0.30	0.82	1.10	0.17	0.32	0.34	1.04	0.76	1.03
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 7.8, (99.9)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 11.4, (99.9)						
cis-Dekalin	1.22	0.97	8.24	11.0	0.85	0.82	1.01	8.93, (99)	6.15, (99)	4.27, (99)
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 69.5, (99.9)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 167, (99.9)						
Mean	2.65	1.76	4.62	5.22	2.58	4.63	1.02	4.23	--	2.66
d.o.f.	56	32	44	24	24	24	8	12	-	4
	Height ratios									
1-Propanol	6.52	4.37	18.9	26.7	2.29	3.70	2.73	20.3, (99)	7.90	15.14, (95)
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 21.4, (99.9)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 49.3, (99.9)						
Butyl acetate	3.01	1.72	3.70	5.00	0.63	1.62	0.72	4.0	3.74	3.98
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 4.8, (99)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 8.95, (99)						
o-Xylene	0.79	0.61	0.81	0.83	0.39	0.34	0.71	0.39	1.47, (90)	0.41
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.77, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 5.86 (95)						
cis-Dekalin	1.29	1.21	16.1	1.01	1.03	0.98	1.12	15.0, (99)	1.89	9.21, (99)
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 162, (99.9)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 1.07, (ns)						
Mean	2.90	1.98	9.87	8.38	1.09	1.66	1.32	9.92,	—	7.19
d.o.f.	56	32	44	24	24	24	8	12	—	4

replication error increases with increasing difference in chemical type from the reference compound. The value for diethyl ether is surprisingly low, which suggests that evaporation losses are negligible for this compound. For the medium-boiling mixture also the value was significantly higher for 1-propanol than for succeeding compounds, the lowest value being obtained for *o*-xylene, which is the most chemically similar of the components to the benzene standard. The high-boiling mixture gave very poor

TABLE VIII

SUMMARY OF RESULTS OF ANALYSIS OF VARIANCE FOR HIGH-BOILING MIXTURE

Reference peak = ethyl benzene (peak 1).

Compounds in order of elution	*Percentage relative standard deviations*									
	Within effects						*Between effects*			
	Reps.	*On-column (oc)*	*Flash v.*	*Set 1*	*Set 2*	*Set 3*	*Temperatures*		*Sets*	*Modes*
							oc	*Flash v.*		
	Area ratios									
(ethylbenzene)										
Tetradecane	13.14	8.34	13.7	6.32	6.92	3.70	0.35	2.34	19.9, (95)	4.01
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 1.86, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 1.73						
Indene	1.02	1.55	0.84	1.57	0.40	0.87	2.36, (95)	0.69	1.92, (90)	0.78
	$F\left(\frac{\text{oc}}{\text{flash}}\right)$ = 3.41, (95)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 3.25, (95)						
Hexadecane	7.96	5.57	7.76	2.87	3.30	1.88	1.09	7.11	32.8, (95)	12.5
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 2.42, (90)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 1.77, (ns)						
Mean	7.37	5.15	7.45	3.59	3.54	2.15	1.27	3.38	–	5.75
d.o.f.	42	24	33	18	18	18	6	9	–	3
	Height ratios									
Tetradecane	12.53	50.5	30.4	43.0	40.0	38.2	86.7, (99)	59.2, (99)	22.5, (90)	49.0, (99)
	$F\left(\frac{\text{oc}}{\text{flash}}\right)$ = 1.76, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right)$ = 0.91, (ns)						
Indene	5.31	4.74	7.43	2.38	8.08	7.29	5.86	10.48, (95)	4.95	3.74
	$F\left(\frac{\text{flash}}{\text{oc}}\right)$ = 2.54, (90)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right)$ = 10.1, (99)						
Hexadecane	16.81	53.2	33.2	44.7	42.4	38.7	91.5, (99)	61.8, (99)	36.4, (95)	43.1, (95)
	$F\left(\frac{\text{oc}}{\text{flash}}\right)$ = 1.73, (ns)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right)$ = 1.30, (ns)						
Mean	11.55	36.13	23.7	33.0	30.2	28.1	61.4	43.8		31.9
d.o.f.	42	24	33	18	18	18	6	9	–	3

area replication with the exception of the indene value. The reason for this was probably the relatively high boiling points of tetradecane and hexadecane, which cause bias errors due to fractionation at the lower vaporizer temperatures. Because the replication variance was poor for this mixture, the significance of these bias errors was lost. The wide-boiling-range mixture gave the highest values for the coefficient of variation for the diethyl ether peak, the first eluted component, and hexadecane, the last eluted component. The lowest values were obtained for ethylbenzene and cyclohexane, *i.e.*, for components similar to the standard and eluted close to it.

Height ratios. For the low-, medium- and high-boiling mixtures, the coefficients of variation for height ratio replicates are not significantly different from the area values except in a few cases. This suggests that peak height measurements are for the most part as precise as the integrator measurements under constant conditions. However, peak height ratios may be less reliable because of the possibility of injection

TABLE IX

SUMMARY OF RESULTS OF ANALYSIS OF VARIANCE FOR WIDE-BOILING-RANGE MIXTURE

Reference peak = *cis*-dekalin (peak 5).

Compounds in order of elution	*Percentage relative standard deviations*									
	Within effects						*Between effects*			
	Reps.	*On-column v. (oc)*	*Flash*	*Set 1*	*Set 2*	*Set 3*	*Temperatures*		*Sets*	*Modes*
							oc	*Flash v.*		
	Area ratios									
Diethyl ether	3.82	1.57	3.82	2.17	1.38	1.63	0.00	0.99	8.30, (95)	2.21
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 6.99$			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right) = 2$, (ns)						
Cyclohexane	1.04	1.29	0.57	0.75	1.12	1.05	0.58	1.05	0.74	1.49
	$F\left(\frac{\text{oc}}{\text{flash}}\right) = 5.0$, (99)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 2$, (ns)						
Toluene	1.40	1.15	1.38	1.15	0.47	1.14	1.13	0.79	3.02, (95)	1.90
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 1.48$, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right) = 1.1$, (ns)						
Ethylbenzene (*cis*-dekalin)	0.69	0.77	0.79	0.54	0.54	0.99	0.39	0.82	0.80	1.61, (95)
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 1.1$, (ns)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 3.25$, (90)						
Tetradecane	2.28	2.19	2.26	2.19	2.65	1.10	2.95	1.24	4.55, (95)	4.93, (95)
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 1.02$, (ns)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right) = 3.75$, (90)						
Indene	17.1	0.90	2.41	2.40	0.95	0.98	4.36, (99)	3.04, (90)	3.68, (95)	5.75
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 7.33$ (99)			$F\left(\frac{\text{Set 1}}{\text{Set 3}}\right) = 6.31$, (95)						
Hexadecane	3.32	2.65	4.69	1.89	3.03	4.51	2.54	3.21	5.42	8.82, (95)
	$F\left(\frac{\text{flash}}{\text{oc}}\right) = 2.90$, (90)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 6.26$, (95)						
Mean	2.04	1.50	2.27	1.58	1.46	1.64	1.71	1.59	–	3.24
d.o.f.	98	72	77	42	42	42	14	21	–	7
	Height ratios									
Diethyl ether	13.62	21.0	9.73	9.28	23.9	14.4	38.8, (99)	3.37	18.2	157, (99.9)
	$F\left(\frac{\text{oc}}{\text{flash}}\right) = 6.67$, (99)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 7.91$, (95)						
Cyclohexane	7.49	16.3	5.15	7.33	12.2	16.7	29.4 (99.9)	6.48	11.7	24.7
	$F\left(\frac{\text{oc}}{\text{flash}}\right) = 12.4$, (99)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 6.17$, (95)						
Toluene	2.18	16.4	7.23	7.67	11.9	7.5	101.5, (99.9)	13.0, (99.9)	4.60, (95)	13.0, (99.9)
	$F\left(\frac{\text{oc}}{\text{flash}}\right) = 6.01$, (99)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 5.73$, (95)						
Ethylbenzene	4.68	12.1	6.06	5.83	9.33	13.1	22.1, (99.9)	11.0, (95)	5.18	14.0, (99)
	$F\left(\frac{\text{oc}}{\text{flash}}\right) = 4.54$, (95)			$F\left(\frac{\text{Set 3}}{\text{Set 1}}\right) = 5.48$, (95)						

TABLE IX *(continued)*

Compounds in order of elution	*Percentage relative standard deviations*									
	Within effects						*Between effects*			
	Reps.	*On-column (oc)*	*Flash v.*	*Set 1*	*Set 2*	*Set 3*	*Temperatures*		*Sets*	*Modes*
							oc	*Flash v.*		
Tetradecane	8.00	27.3	23.3	25.9	26.5	25.8	50.3, (99.9)	44.2, (99.9)	4.80	21.4, (95)
	$F\left(\frac{oc}{flash}\right) = 1.13$, (ns)			$F\left(\frac{Set\ 3}{Set\ 1}\right) = 1.06$, (ns)						
Indene	7.35	14.3	10.1	7.23	12.3	16.4	24.1, (99)	9.47	6.52	12.7
	$F\left(\frac{oc}{flash}\right) = 2.22$, (ns)			$F\left(\frac{Set\ 3}{Set\ 1}\right) = 5.59$, (95)						
Hexadecane	7.24	29.0	21.1	21.2	29.6	26.8	53.8, (99.9)	42.6, (99.9)	3.33	19.4, (95)
	$F\left(\frac{oc}{flash}\right) = 1.58$, (ns)			$F\left(\frac{Set\ 3}{Set\ 1}\right) = 1.68$, (ns)						
Mean	7.22	19.5	11.8	12.1	18.0	18.7	45.7	18.6	—	37.4
d.o.f.	98	72	77	42	42	42	14	21	—	7

function effects. For instance, diethyl ether, the first eluted component of both the low- and wide-boiling-range mixtures, and hexadecane in the high-boiling mixture gave poor replicate height ratios, suggesting the existence of adverse injection function effects. The wide-boiling-range mixture gave much poorer replication for heights than for areas for all the components, indicating that injection function effects are more critical than for the other mixtures.

On-column and flash vaporization

Area ratios. For the low- and medium-boiling mixtures the precision in the on-column mode is better than the precision in the flash vaporization mode, the difference being significant, or very significant, in a number of cases. With the high-boiling mixture both tetradecane and hexadecane give better precisions for on-column injections than for flash vaporization but indene gave a significantly inferior precision in the on-column mode. This is probably due to a slight polymerization of indene. With the wide-boiling-range mixture only diethyl ether, indene and hexadecane gave significantly better precisions for on-column injection than for flash vaporization.

Height ratios. The precision of on-column injection using height ratios is significantly better than the precision of flash vaporization in all cases except for the high-boiling mixture where the reverse situation applies. The latter anomaly is explained by the relatively low vaporizer temperatures compared with the boiling points of the components, an example of poor chromatographic practice.

Sets

Area ratios. The precision of set 1 is significantly worse than the precisions of sets 2 and 3 for most compounds, except for the wide-boiling-range mixture. It seems unlikely that this difference in precision is due to the particular type of syringe used because the same type was also used for half of the injections of set 2. The most likely

explanation is that the syringe used for the injections of set 1 was badly worn in comparison with the other syringes thus giving rise to a poorer precision for the set. This syringe was, in fact, changed for the wide-boiling-range mixture, which probably explains why set 3 tends to give a poorer precision than sets 1 and 2 in this case.

Height ratios. The effects obtained for the within set height ratios are much greater than the area ratio effects for the low-boiling mixture. This is almost entirely due to poor injection functions arising from an occasional poor injection by the operator. For the medium-boiling mixture, however, the results are similar to those for the area ratios, set 1 again being poorer than sets 2 and 3. With the high- and wide-boiling-range mixtures, there are considerable injection function effects, particularly for the latter where all the values are much worse than for areas.

Between effects

Vaporizer temperature

Area ratios. In general, the level of the vaporizer temperature had no significant effect on the relative peak areas, which strongly suggests that the sample entering the column was representative of the original sample, within the precision of the procedure. Only indene gave a significant effect, again probably due to polymerization.

Height ratios. In contrast to the lack of significant effects for area ratios, the height ratios gave a number of significant effects. These arose in the use of flash vaporization with the low- and medium-boiling mixtures and in both modes with the high- and wide-boiling-range mixtures. These effects were almost entirely due to variable peak spreading induced by injection function effects. For the wide-boiling-range mixture in particular, the effect was very significant for nearly all components.

Sets

Area ratios. There are numerous significant differences between sets except where the compounds concerned are chemically similar to the reference compound. These differences are clearly explained by long-term changes in the relative response of the equipment, probably in the detector. When the measured compound is similar to the reference compound (toluene in the low-boiling mixture and *o*-xylene in the medium-boiling mixture), the response changes would tend to be compensatory. With the wide-boiling-range mixture there is evidence of changes due to evaporation losses, but here again the compound most chemically similar to the *cis*-dekalin reference, *i.e.* cyclohexane, gives the least effect.

Height ratios. With the low-, medium- and high-boiling mixtures the set height variances are similar to the area variances and so they probably arise from the same sources. With the wide-boiling-range mixture, diethyl ether, cyclohexane and ethylbenzene give much higher values than for areas and so clearly there is an additional injection function effect.

Modes

Area ratios. Apart from one or two exceptions, there is no significant difference between on-column and flash vaporization injection with regard to peak area ratios. Thus, again, it seems highly improbable that there are any significant bias errors in the injection procedure. With the medium-boiling-mixture *cis*-dekalin gave

a significant effect because of a particularly poor injection that resulted in severe fractionation from the needle. For the wide-boiling-range mixture there were significant differences for ethylbenzene, tetradecane and hexadecane, which indicated the existence of bias errors in these cases. On inspection it is clear that these errors arise from fractionation effects in the flash vaporization mode as described earlier.

Height ratios. There are significant or highly significant effects for methanol and toluene in the low-boiling mixture, 1-propanol and *cis*-dekalin in the medium-boiling mixture, and for all components except indene in the wide-boiling-range mixture. All these differences are primarily due to greater peak spreading when using the on-column mode than when using flash vaporization.

GENERAL CONCLUSIONS

The statistical experiments described in this paper indicate that bias or random errors can arise from the sample injection process both as a result of operator technique and instrumental conditions. The results suggest that bias errors are not likely to be significant if a sound code of chromatographic practice is followed, but random errors, which affect the precision of peak area ratios, depend critically on both technique and injection conditions. Clearly the operator's injection technique should be carefully standardized, preferably on the lines described, and the vaporizer temperature should be sufficiently high to avoid slow vaporization or an adverse injection function.

In general, for low- and medium-boiling mixtures, on-column injection gave better precision and fewer bias errors for both peak height and area measurements than flash vaporization. The latter, however, tended to give a better injection function and hence a better precision for peak heights for components covering a wide boiling range. An examination of the mean values indicates that the best compromise would be to use on-column injection with additional heat supplied by a vaporizer.

Concerning the choice of reference compound to be used as an internal standard, maximum precision was obtained for components similar in chemical type and eluted closest to the standard.

REFERENCE

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