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EFFECT OF OVERLOAD OF CAPILLARY GAS–LIQUID CHROMATOGRAPHIC COLUMNS ON THE EQUIVALENT CHAIN LENGTHS OF C_{18} UNSATURATED FATTY ACID METHYL ESTERS

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SUMMARY

Column overload causes errors in the estimation of equivalent chain lengths (ECLs) of fatty acid methyl esters (FAMEs), and the extent of these errors on fused-silica open tubular columns has been investigated. Load limits for several common FAMEs were accurately determined utilizing a synchronized, rapid automatic liquid sampler. The findings were applied to obtain estimates of ECLs of a range of C_{18} unsaturated FAMEs to a precision (repeatability) of about ± 0.001 ECL units. Three stationary phases, DEGS, SP-2330 and Supelcowax 10, were studied, each at three temperatures.

INTRODUCTION

The equivalent chain length (ECL), a concept first developed by Woodford and Van Gent[1] and by Miwa *et al.*[2], conveniently expresses the retention properties of a fatty acid methyl ester (FAME) in a form which allows its position in a chromatogram to be readily visualized in relationship to nearby saturated, straight-chain FAMEs or, conversely, for an unknown FAME in a chromatogram to be tentatively identified. While ECL is defined according to eqn. 1, its determination in practice presents a number of well-debated problems.

$$\mathrm{ECL} = n + \frac{\log t'_{\mathrm{R},x} - \log t'_{\mathrm{R},n}}{\log t'_{\mathrm{R},n+1} - \log t'_{\mathrm{R},n}} \quad (1)$$

where $t'_{\mathrm{R},x}$ = corrected retention time of unknown FAME x; $t'_{\mathrm{R},n}$ = corrected retention time of nearest saturated, straight chain FAME eluting ahead of x; $t'_{\mathrm{R},n+1}$ corrected retention time of next higher homologue of n; and $t'_{\mathrm{R}} = t_{\mathrm{R}} - t_{\mathrm{M}}$, where t_{R} = uncorrected retention time; and t_{M} = column dead time. Errors in the determination of ECL may result from two sources. First, there are errors resulting from purely chromatographic phenomena which include errors in the determination of the column dead time, t_{M}[3–5], errors resulting from the retention time relationship for members of an homologous series shown in eqn. 2 which, according to some work-

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ers[6,7], may not be strictly linear, and errors resulting from peak overlap or column overload[8–12].

$$\log t'_R = aN + b \qquad (2)$$

where a and b are constants and N is the number of carbon atoms in the major chain of the molecule. Errors in the determination of ECL may also result from external physical variables such as type, variability and stability of the stationary phase[13,14] column oven temperature and its stability[13,15,16] and interaction with the solid support in the case of packed columns, or with the wall material in the case of capillary columns[17].

The problem of column overload has not been extensively discussed, probably because much of the earlier work was carried out on packed columns where overload is not a significant problem. However, wall-coated open tubular columns, especially those with thin stationary phase films, are highly vulnerable to overload, which results in error due to retardation of retention times. Ackman and Castell[8] showed that, in the case of monenoic FAME, a large amount of an earlier eluting isomer displaced other isomers of longer retention times from their normal positions, provided that the isomers were of similar structure. In a later paper, Ackman and Hooper[9] showed that structurally dissimilar materials, such as *trans* acids in a mixture of *cis* isomers, did not exhibit this "load effect" and proposed that the displacement was due to mutual exclusion of structurally very similar materials through saturation effects in the partitioning between liquid and gas planes. This phenomenon was further discussed by Ackman[10] and by Ackman and Eaton[11]. Gillan[12] recognized column overload as problem and developed a mathematical model to reduce the retention time of overloaded peaks in capillary columns to a "standard retention time" and also a polynomial equation to correct ECL estimates. In the present work we have further investigated the problem of overload to improve the accuracy of ECL estimation. An important tool in this work has been the use of a synchronized, rapid automatic liquid sampler for the very accurate estimation of retention times, and we report ECLs of improved precision for a wide range of unsaturated C_{18} FAMEs determined on three fused-silica open tubular (FSOT) columns, each at three temperatures.

EXPERIMENTAL

Isooctane (2,2,4-trimethylpentane) was Pronalys analytical reagent grade (May and Baker, West Footscray, Australia). Reference saturated esters were methyl palmitate, methyl stearate and methyl arachidate (Sigma, St. Louis, MO, U.S.A.). Reference octadecenoic acid methyl esters had the double bond configurations *cis*-4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, *trans*-5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, and -17 [Hormel Institute, Austin, MN, U.S.A., except *cis*-9 (Sigma) and *cis*-6; *trans*-6 and *trans*-9 (Alltech, Deerfield, IL, U.S.A.)]. Reference octadecadienoic acid methyl esters had the double bond configurations *cis*-9, *cis*-12 (Sigma); *trans*-8, *trans*-12; *cis*-9, *trans*-12; *trans*-9, *cis*-12; *trans*-9, *trans*-12; *trans*-9, *trans*-13; *trans*-9, *trans*-15 (Unilever Research Laboratory, Vlaardingen, The Netherlands). The octadecatrienoic acid ester was methyl linolenate (Sigma). For simplicity we use 18:1 to mean methyl oleate (*cis*-9), 18:2 to mean methyl linoleate (*cis*-9, *cis*-12) and 18:3 to mean methyl linolenate (*cis*-9, *cis*-12, *cis*-15).

Apparatus

Gas–liquid chromatography (GLC) was carried out on a Hewlett-Packard Model 5880A gas chromatograph fitted with a flame ionization detector, a split/-splitless capillary inlet system and a Model 7673A rapid automatic liquid sampler. The sampler was synchronized with a Hewlett-Packard Model 3350A laboratory automation system which was used to measure retention times and peak areas. The injection volume was 1 μl. The columns were 22 m × 0.22 mm I.D. FSOT coated with 0.2 μm film thickness of DEGS (Chrompack, Middelburg, The Netherlands), 60 m × 0.25 mm I.D. FSOT coated with 0.2 μm of SP-2330 (Supelco, Bellefonte, PA, U.S.A.) and 50 m × 0.35 mm I.D. FSOT coated with 0.25 μm of Supelcowax 10 (Supelco).

The inlet system was used in the split mode and was fitted with a standard Hewlett-Packard glass liner of the type described by Jennings[18]. The injector temperature was 375°C for DEGS and 300°C for SP-2330 and Supelcowax 10. The carrier gas was hydrogen (99.995%) with the inlet pressure adjusted for the various columns to give a carrier gas linear velocity of 35–45 cm/s. The pressures were 10 p.s.i. for DEGS, 22 p.s.i. for SP-2330 and 12 p.s.i. for Supelcowax 10. The septum purge flow-rate was *ca.* 3 ml/min. The split vent flow-rate was varied to give a split ratio of approximately 140:1 for each column in order to provide comparable peak masses in the column overload experiments described below. The split vent flow-rates were 160 ml/min for DEGS, 150 ml/min for SP-2330 and 250 ml/min for Supelcowax 10. Three oven temperatures were used for each phase and were 150, 160 and 170°C for DEGS, 180, 200 and 220°C for SP-2330 and 200, 220 and 240°C for Supelcowax 10. Supplementary hydrogen was supplied to the detector to give a total flow-rate of 30 ml/min, the make-up gas was nitrogen (99.995%) and had a flow-rate of 24 ml/min, and oil-free, compressed air was supplied at a flow-rate of 240 ml/min. The detector temperature was 250°C.

Calculation of ECLs

ECLs were determined by co-injecting a mixture of the FAMEs 16:0, 18:0 and 20:0 with the unsaturated ester. The mathematical dead time of the column was first calculated by the method of Peterson and Hirsch[19] as modified by Hafferkamp[20] and Hansen and Andresen[21] using the uncorrected retention times of 16:0, 18:0 and 20:0 according to eqn. 3.

$$t_M = \frac{t_{18}^2 - (t_{16}t_{20})}{2t_{18} - t_{16} - t_{20}} \tag{3}$$

where t_M = mathematical dead time; t_{16} = uncorrected retention time of 16:0; t_{18} = uncorrected retention time of 18:0; t_{20} = uncorrected retention time of 20:0. The ECL of the unsaturated ester x was then calculated according to eqn. 4 which is a modification of eqn. 1 to suit the present investigation, using the retention times of 16:0, 20:0 and of the unsaturated ester, all corrected with respect to the mathematical dead time.

$$\mathrm{ECL}_x = 16 + \frac{4(\log t'_x - \log t'_{16})}{\log t'_{20} - \log t'_{16}} \tag{4}$$

where t'_x = corrected retention time of the unsaturated ester = $t_x - t_M$; t'_{16} = corrected retention time of 16:0 = $t_{16} - t_M$; t'_{20} = corrected retention time of 20:0 = $t_{20} - t_M$. The precision (repeatability) of ECL determinations was estimated by carrying out ten consecutive injections and calculating the standard deviation and coefficient of variation.

Estimation of column load limits

In order to investigate the effect of column overload on ECL, the load limits for the esters 16:0, 18:0, 18:1, 18:2, 18:3 and 20:0, which are referred to herein as the reference esters, were established by preparing mixtures such that, in a given mixture, each of these esters had the same concentration, with this concentration being progressively increased for each of the mixtures. The concentrations of each component in the individual mixtures were 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.5 and 1.0%. For a given set of chromatographic conditions on a column, 1-μl injections of each mixture were made consecutively in increasing order of concentration, with the 0.005% mixture being injected again at the end to determine if systematic changes in any of the retention times had occurred since the initial injection. Peak retention times were observed as a function of peak area, and the column load limit for each peak was specified in terms of a maximum peak area at which no significant retardation of retention time was observed, *i.e.* the peak had not started to develop the skewed leading edge characteristic of overloaded peaks. These areas were also converted to absolute mass loads using the split ratio. It was assumed that the various mono- and dienoic FAMEs which were later studied had load limits similar to those of 18:1 and 18:2 respectively.

Determination of ECLs of unsaturated FAMEs

Having established load limits for the various reference FAMEs, each of the unsaturated FAMEs were in turn co-injected with a mixture of the esters 16:0, 18:0 and 20:0 under conditions which did not exceed these limits and the ECL calculated according to eqns. 3 and 4. In order to monitor the validity of the ECL determinations, a mixture of the reference FAME 16:0, 18:0, 18:1, 18:2, 18:3 and 20:0 was injected ten times initially to establish the status of the system and thereafter at frequent intervals while the unsaturated esters were being investigated. The ECLs of 18:1, 18:2 and 18:3 thus determined were used to detect fluctuations in the effective polarity of the column being examined. Minor fluctuations were in fact observed for all three columns, and corrections, which were rarely greater than 0.001 ECL units, were made to estimates of the ECLs of the various unsaturated FAMEs according to the method of Scholfield[22], the principle of which is shown in eqn. 5 for a monoenoic FAME.

$$\mathrm{ECL}_x(\mathrm{corrected}) = \mathrm{ECL}_x(\mathrm{observed}) + \mathrm{ECL}_{18:1}(\mathrm{average}) + \\ - \mathrm{ECL}_{18:1}(\mathrm{observed}) \qquad (5)$$

where $\mathrm{ECL}_{18:1}$(average) = average ECL of 18:1 over the duration of the experiment; $\mathrm{ECL}_{18:1}$(observed) = ECL of 18:1 determined as near as possible in time to the estimate of ECL_x. A similar correction was applied to dienoic FAMEs using corresponding ECLs for 18:2.

RESULTS AND DISCUSSION

Calculation of ECLs

The method used to calculate an ECL can itself affect the accuracy and precision of the estimate obtained. The first source of error lies in the determination of the column dead time, t_M, which itself may be estimated experimentally by measuring the retention time of methane or mathematically from the retention properties of selected reference FAMEs. Dorris *et al.*[13] have discussed these problems pointing out that although some workers[3] believe that the retention time of methane is a good approximation of t_M' others[4,5] maintain that methane is significantly retained on most stationary phases. They demonstrated that the use of the mathematical dead time did in fact produce more accurate ECL values than those using the retention time of methane, the major drawback of the use of the mathematical dead time being the assumption that the relationship shown in eqn. 2 is linear. Wainwright and Haken[23] have reviewed methods for the calculation of the mathematical dead time and have also severely questioned the use of methane to estimate column dead time.

The second source of error in calculating ECL is the assumption, already pointed out above, that eqn. 2 may not be strictly linear. While there is evidence to support this non-linearity[6,7] the effect can be minimized by the choice of a suitable technique. In order to minimize both the above sources of error we have used the mathematical dead time defined by eqn. 3 for our own determinations. This equation necessarily gives an exact determination of ECL for 18:0 in eqn. 4 and, accordingly, would be expected to give very accurate estimates for other peaks in this vicinity, which is our main areas of interest.

Estimation of peak load limits

In order to carry out the column load limit experiments it was essential that the columns gave highly reproducible retention times under a given set of conditions, as the criterion for detecting the point of overload was that the retention time of the ester was significantly retarded when this point was reached. Accurate determination of the retention times required, first, that the timing device be started in a very reproducible manner and, second, that systematic drift of retention times was not significant. The first problem was overcome by the use of the synchronized rapid automatic liquid sampler. The second was overcome by allowing the column to stabilize over a long period of time, say, overnight. To verify that the column was stable, it was required that a reproducible retention time was obtained when the sample which introduced the lowest load (0.005% solution) was injected at the start and at the end of an experimental run.

The results of the column load limit experiment which was carried out on the SP-2330 column at 200°C are given in Table I. Similar experiments were carried out on the DEGS and Supelcowax 10 columns, but are not reported here because of the essentially similar findings and conclusions. Several conclusions were drawn from the results in Table I. First, the stability of the system and, in turn, the validity of the experiment was indicated by the close similarity of the values obtained respectively for retention times, peak areas and ECLs when the 0.005% solution was analysed at the beginning and end of the series. Second, the synchronized rapid injector/computer system gave high accuracy of determination of retention times. this conclusion also

TABLE I

RETENTION TIMES, RAW PEAK AREAS AND ECL VALUES OF SELECTED FAMEs AS A FUNCTION OF SAMPLE SIZE INJECTED

Concentration of each ester in sample (%) 1 µl injection	*Approximate peak mass (ng)*	*Retention time (min)*						*Raw peak area*						*ECL*		
		16:0	*18:0*	*20:0*	*18:1*	*18:2*	*18:3*	*16:0*	*18:0*	*20:0*	*18:1*	*18:2*	*18:3*	*18:1*	*18:2*	*18:3*
0.005	0.04	4.291	5.163	6.593	5.569	6.237	7.184	449	472	441	464	530	497	18.675	19.587	20.605
0.01	0.07	4.291	5.163	6.591	5.569	6.236	7.182	704	752	683	735	802	800	18.675	19.587	20.606
0.025	0.18	4.292	5.164	6.594	5.570	6.237	7.184	1588	1672	1620	1648	1720	1806	18.675	19.586	20.604
0.05	0.4	4.294	5.168	6.601	5.575	6.242	7.190	3383	3515	3430	3475	3634	3783	18.674	19.584	20.601
0.1	0.7	4.293	5.168	6.603	5.574	6.242	7.190	6185	6429	6297	6358	6564	7047	18.673	19.582	20.599
0.2	1.5	4.295	5.173	6.613	5.579	6.247	7.196	12 617	13 320	12 869	13 176	12 615	11 570	18.671	19.578	20.593
0.5	4	4.298	5.182	6.635	5.588	6.258	7.208	32 037	33 810	32 541	33 305	31 999	29 384	18.660	19.568	20.578
1.0	7	4.304	5.196	6.661	5.602	6.273	7.226	66 366	69 764	67 237	68 801	65 696	69 677	18.660	19.558	20.566
0.005	0.04	4.293	5.167	6.598	5.573	6.241	7.189	442	467	446	465	527	497	18.675	19.586	20.605

being supported by the results given later in Table II. Third, because of the high accuracy of retention time determinations, it was easy to detect even the smallest retardation of retention time, indicative of peak skewing due to column overload. Fourth, sensitivity to overload increased with increasing chain length, which is, of course, only a manifestation of the well known phenomenon that any compound will elute as a skewed (leading) peak as the temperature is reduced. These limits corresponded to absolute loads of approximately 15 ng (0.2% solution) for 16:0 and approximately 2 ng (0.025% solution) for 20:0. Fifth, under conditions of overload, the ECLs of unsaturated esters were underestimated, first, because the 20:0 peak was retarded to a greater extent than the 16:0 peak and, second, because the unsaturated esters were retarded less than the corresponding saturated ester, *viz.* 18:0. This is consistent with Ackman's comment[10] that, as the unsaturation of the FAME increases, its susceptibility to overload decreases. It may be further noted that the conclusions that we have drawn from this work carried out on thin film FSOT columns are in general agreement with those variously made by earlier workers who used steel columns that were frequently more heavily coated. It would be of interest to determine whether the generality of the phenomena is such as to extend also to the "megabore" columns now in common use.

The above results enabled a load specification to be selected for the valid determination of ECLs in terms of a maximum raw peak area which was set empirically at 1000 counts for the system being used.

Accuracy of retention time and ECL determinations

It has already been indicated above that systematic drift in retention times normally occurred unless the columns were well stabilized. As it was impractical to achieve such stability all of the time, the reproducibility of ECL determinations was examined under realistic conditions *viz.*, after baselines were allowed to stabilize, but

TABLE II

RETENTION TIMES, ECLs AND STATISTICAL RESULTS FOR 10 CONSECUTIVE DETERMINATIONS FOR 18:3 ON SP-2330 AT 180°C

Retention time (min)	*ECL*
11.737	20.3879
11.762	20.3881
11.779	20.3880
11.802	20.3873
11.836	20.3869
11.847	20.3876
11.853	20.3872
11.861	20.3863
11.864	20.3860
11.859	20.3870
Mean	20.3872
Standard deviation	0.00069
Coefficient of variation	0.0034

no other precautions against systematic drift taken. Results illustrating a typical rate of drift and the repeatability of the ECL of 18:3 under these conditions are given in Table II. The column was the SP-2330 at 180°C, and the load limit was not exceeded. Similar studies were carried out for all of the unsaturated reference esters for each of the columns and oven temperatures, but are too extensive and the results too similar to report here. The results given in Table II illustrate typical rates of drift of retention times encountered under normal operating conditions during the experiments. More importantly, this drift had little effect on the calculated ECL value of 18:3, as the estimates of the retention times of the reference saturated esters and of t_M also drifted and this resulted in potential errors being largely cancelled out. As a result it was found possible to estimate ECLs with a precision of about ±0.001 compared with errors reported in the literature which are most commonly in the range ±0.01 to ±0.04[13,22,24]. It was concluded that the technique used in the experiments, especially the precautions taken to avoid column overload, had been effective in significantly improving both the accuracy and the precision of ECL estimation.

ECLs of unsaturated FAMEs

The results of the ECL determinations for the various unsaturated FAMEs are given in Table III. These results provide a database which is more comprehensive and more precise than those which have appeared in the literature to date[16,25–33]. It may accordingly be used for the more reliable identification of unknown FAMEs or for selecting improved conditions for the separation of particular FAMEs, providing certain inherent problems are dealt with. The first difficulty lies in dealing with column overload, which must be avoided, if accurate comparisons of ECLs are to be made. Because of the widely differing concentrations of fatty acids in most practical mixtures, it may be necessary to run the sample at a number of different sample sizes or, alternatively, it may be possible to apply corrections using mathematical techniques such as those of Gillan[12]. Similar techniques could also possibly be used to deal with peak overlap.

A more serious difficulty lies in relating data such as those given in Table III to those obtained on nominally similar columns. This problem arises out of stationary phase variability and instability which, of all the parameters relevant to the estimation of ECLs, are the two that are likely to present the most intractable problems. Thus, the vast majority of useful phases available at the present time are mixtures which vary in composition, albeit slightly, from batch to batch and which may be subject to change by way of bleeding and reactions such as further polymerization, oxidation, reaction with analytes and the like. While the results given in Table III were very reproducible over a time frame of several weeks, small, but significant changes were sometimes found after a time frame of several months, which we ascribe to marginal polarity changes in the phase. This behaviour does not detract from the significance of the results given above which represent very accurate estimates of the relative locations of the various unsaturated esters under conditions which remained very stable for each of the various sets of parameters. It should be possible to correlate such data accurately to those obtained on similar, if not absolutely identical phases at the stated temperatures by determining ECLs for one or more of the commonly available unsaturated esters and applying corrections accordingly.

TABLE III

ECLs OF UNSATURATED C_{18} FAMEs

FAME	*ECL DEGS*			*SP-2330*			*Supelcowax 10*		
	Column temperature (°C)			*Column temperature (°C)*			*Column temperature (°C)*		
	150	*160*	*170*	*180*	*200*	*220*	*200*	*220*	*240*
18:1:-									
cis-4	18.318	18.348	18.376	18.394	18.453	18.511	18.225	18.245	18.264
cis-5	18.217	18.254	18.290	18.393	18.473	18.551	18.147	18.178	18.208
cis-6	18.325	18.363	18.399	18.534	18.616	18.695	18.217	18.245	18.271
cis-7	18.294	18.333	18.372	18.524	18.612	18.698	18.192	18.222	18.252
cis-8	18.305	18.348	18.387	18.548	18.639	18.729	18.202	18.233	18.264
cis-9	18.334	18.377	18.416	18.584	18.675	18.765	18.223	18.254	18.283
cis-10	18.365	18.407	18.446	18.619	18.710	18.801	18.250	18.281	18.309
cis-11	18.412	18.453	18.492	18.667	18.757	18.845	18.291	18.320	18.347
cis-12	18.479	18.521	18.557	18.731	18.819	18.906	18.350	18.377	18.403
cis-13	18.551	18.591	18.629	18.801	18.887	18.973	18.417	18.442	18.466
cis-14	18.633	18.673	18.712	18.875	18.959	19.043	18.497	18.519	18.542
cis-15	18.728	18.768	18.805	18.937	19.016	19.095	18.579	18.588	18.618
cis-16	19.053	19.102	19.147	19.286	19.383	19.479	18.896	18.919	18.942
trans-5	18.301	18.329	18.352	18.333	18.379	18.415	18.231	18.248	18.261
trans-6	18.321	18.350	18.372	18.383	18.428	18.467	18.236	18.250	18.262
trans-7	18.302	18.332	18.359	18.390	18.441	18.485	18.221	18.239	18.254
trans-8	18.308	18.339	18.366	18.396	18.450	18.501	18.226	18.244	18.259
trans-9	18.317	18.348	18.375	18.423	18.475	18.522	18.228	18.248	18.263
trans-10	18.345	18.377	18.403	18.448	18.501	18.549	18.252	18.268	18.284
trans-11	18.373	18.403	18.430	18.471	18.523	18.573	18.274	18.291	18.304
trans-12	18.414	18.442	18.467	18.517	18.566	18.613	18.306	18.319	18.331
trans-13	18.461	18.490	18.513	18.548	18.592	18.635	18.346	18.358	18.366
trans-14	18.478	18.505	18.526	18.555	18.595	18.629	18.357	18.366	18.371
trans-15	18.574	18.596	18.618	18.618	18.651	18.680	18.433	18.436	18.435
trans-16	18.824	18.857	18.886	18.862	18.905	18.949	18.661	18.666	18.667
Δ-17	18.7345	18.771	18.805	18.910	18.981	19.052	18.561	18.573	18.586
18:2:-									
trans-8,*trans*-12	18.718	18.770	18.816	18.866	18.959	19.048	18.509	18.534	18.560
cis-9,*cis*-12	18.995	19.069	19.131	19.429	19.586	19.748	18.688	18.733	18.777
cis-9,*trans*-12	19.003	19.067	19.123	19.266	19.390	19.511	18.730	18.763	18.793
trans-9,*cis*-12	18.957	19.007	19.050	19.087	19.166	19.244	18.701	18.716	18.730
trans-9,*trans*-12	18.959	19.008	19.050	19.087	19.169	19.245	18.700	18.717	18.730
trans-9,*trans*-13	18.773	18.824	18.869	18.921	19.012	19.100	18.554	18.578	18.600
trans-9,*trans*-15	18.926	18.975	19.019	19.059	19.142	19.221	18.674	18.689	18.703
trans-10,*trans*-12	20.529	20.619	20.699	21.062	21.228	21.391	20.140	20.170	20.199
18:3	19.848	19.948	20.039	20.387	20.606	20.834	19.326	19.385	19.441

CONCLUSIONS

The effect of column overload on the accuracy of estimating the ECLs of FAMEs has not been extensively studied to date. This effect is very significant in the case of capillary columns and probably accounts in part for many of the discrepancies seen in the literature. If due precautions are taken, estimates of ECL may be made with a precision of about ± 0.001 ECL units.

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