ELSEVIER

Journal of Chromatography A, 803 (1998) 1–31

JOURNAL OF CHROMATOGRAPHY A

Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development
I. Application to 14 different samples using computer simulation

J.W. Dolan[a,*], L.R. Snyder[a], N.M. Djordjevic[b], D.W. Hill[c], D.L. Saunders[d], L. Van Heukelem[e], T.J. Waeghe[f]

[a]*LC Resources Inc., 2930 Camino Diablo, Walnut Creek, CA 94596, USA*
[b]*Novartis Pharma AG, Basel 4002, Switzerland*
[c]*Microchemistry Laboratory, University of Connecticut, Storrs, CT 06269, USA*
[d]*Avery Dennison, Pasadena, CA 91107, USA*
[e]*Horn Point Lab., UMCES, P.O. Box 775, Cambridge, MD 21613, USA*
[f]*DuPont Co., Experimental Station, P.O. Box 8040, Wilmington, DE 19880, USA*

Received 13 October 1997; received in revised form 22 December 1997; accepted 23 December 1997

Abstract

The optimized reversed-phase HPLC separation of 14 different samples is reported, based on simultaneous changes in temperature and gradient steepness. Four experimental runs are required for each sample, following which preferred conditions can be predicted using computer simulation software (DryLab). The overall accuracy and effectiveness of this method development approach is discussed, with particular attention to the use of resolution maps provided by the software. These maps are useful for maximizing resolution for the total sample, for optimizing the separation of a smaller number of selected sample compounds, and as an initial step in the separation of more demanding samples. © 1998 Elsevier Science B.V.

Keywords: Computer simulation; Temperature effects; Gradient elution; Optimization; Resolution; Method development; Steroids; Pesticides; Algal pigments; Fatty acid methyl esters; Acrylate monomers

1. Introduction

Previous work [1–3] for some widely different samples suggests that changes in temperature (T) and/or gradient steepness (b) are an effective means of changing band spacing (selectivity or α) and improving sample resolution. While changes in either T or b have a smaller effect on selectivity than changes in solvent type or pH [4], this is less true when T and b are *simultaneously* optimized for a given sample. The latter approach, when combined with the use of acetonitrile–buffer mobile phases, has several potential advantages when compared to alternative method development procedures [3–6]: (a) simpler, more convenient method development – especially with the use of computer simulation as

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01293-4

described here, (b) more troublefree and robust final methods, (c) fewer detection problems with UV detection, (d) faster column equilibration when changing from one method to another, and (e) applicability to both neutral and ionized samples (mixtures of acids and/or bases).

A major question concerning method development based on changes in T and b is whether this approach is likely to be successful for a given sample. A prior analysis [4] suggests that compounds whose polar substituents differ in terms of type and/or number are more likely to be separable by optimizing T and b. Conversely, isomeric compounds or samples composed of molecules with few or no polar substituents are less likely to be separated in this way. A further question is how to proceed when the optimization of T and b is unsuccessful in initial attempts at separating a given sample. These and other questions are addressed in the present and following paper. Some advantages of computer simulation for optimizing T and b will also be illustrated.

2. Theory

The theory of computer simulation for predicting separation as a function of different experimental conditions and either isocratic or gradient elution has been reviewed [7–11]. This treatment has been extended for the simultaneous change of gradient steepness and temperature, and shown to be adequately reliable [5]. For reversed-phase separation, it is assumed that isocratic retention factors k for a given solute are given by

$$\log k = \log k_w - S\varphi \quad (1)$$

where k_w is a constant, corresponding to a value of k extrapolated to water as mobile phase ($\varphi = 0$), φ is the volume fraction of organic in the mobile phase ($\varphi = 0.01\%$ B), and S is a constant for a particular solute. Retention times t_R in gradient elution can be calculated [7,9,11] from values of k_w and S for each solute in the sample and gradient steepness b, where

$$b = V_m \Delta\varphi S/(t_G F) \quad (2)$$

V_m refers to the column dead volume (ml), $\Delta\varphi$ is the change in φ during the gradient, t_G is gradient time (min) and F is flow-rate (ml/min). Retention times in gradient elution have been related to temperature [5] by the empirical relationship

$$t_R = A + BT \quad (3)$$

where A and B are constants for a given compound when only temperature T is varied. Eq. (3) appears to be less reliable for predictions of t_R when $T <$ 25°C and/or for polymeric alkyl-silica column packings [5]. For the latter cases, more accurate predictions are possible if a restricted range in temperature is explored (e.g., 10–20°C), or if additional experiments are carried out for more than two temperatures.

3. Experimental

3.1. Materials and procedures

Results are presented here from several different laboratories, based on data for new samples and previously described samples whose separation has so far not been optimized by means of computer simulation.

3.1.1. Laboratory A (Djordjevic)

Experimental conditions for these separations were as described in Ref. [5], unless noted otherwise. The samples studied were a synthetic organic mixture (No. 2 of Table 2), a pharmaceutical mixture (No. 9 of Table 2) and a mixture of LSD derivatives (No. 13 of Table 2).

3.1.2. Laboratory B (Hill)

Experimental conditions for these separations were as described in Ref. [5], unless noted otherwise. The equipment hold-up (''dwell'') volume was 5.5 ml for the corticosteroid separations of Fig. 7, and 1.15 ml for the testosterone separations of Fig. 8. The samples studied were mixtures of corticosteroids (No. 6 of Table 2), testosterones (No. 7 of Table 2) and toxicology standards (No. 10 of Table 2).

3.1.3. Laboratory C (Lin)

Experimental conditions for these separations were as described in Ref. [2], unless noted otherwise. The

sample studied was a mixture of fatty acid methyl esters (No. 5 of Table 2).

3.1.4. Laboratory D (Saunders)

A Model HP1050 high-performance liquid chromatography (HPLC) system (Hewlett-Packard) was used with a 30×0.39 cm Novapak C_{18} column (Waters). A dwell volume of 4.0 ml was assumed. The sample studied was a mixture of acrylate monomers (No. 3 of Table 2).

3.1.5. Laboratory E (Van Heukelem)

Experimental conditions for these separations were as described in Ref. [2], unless noted otherwise. Two columns were used: (a) a 25×0.32 cm Vydac 201tp53 C_{18} ''polymeric'' column (The Separations Group) and (b) a 10×0.21 cm Hypersil ODS C_{18} ''monomeric'' column (Shandon); solvent A was 70% methanol, 28 m*M* aqueous tetrabutyl ammonium hydroxide adjusted to pH 7.1 with acetic acid; solvent B was methanol. The sample studied was a mixture of algal pigments (No. 4 of Table 2).

3.1.6. Laboratory F (Waeghe)

Experimental conditions for these separations were as described in Ref. [5], unless noted otherwise. The column was a 25×0.46 cm Zorbax SB-C_8 column (5-μm particles) from MacMod Analytical (Chadds Ford, PA, USA). The samples studied were mixtures of herbicides (No. 1 of Table 2) or herbicide impurities (No. 8 of Table 2).

3.2. Computer simulation

All simulations reported here were carried out with DryLab for Windows, version 2.0 (LC Resources).

4. Results

A previous report [3] described the separation of four different samples by simultaneously optimizing temperature and gradient steepness. It was possible to obtain baseline resolution for three of these samples, while only partial resolution ($R_s = 0.8$) of the remaining sample was achievable with a 25-cm C_{18} column (5-μm particles). Computer simulation was used in the study of [3], but at that time only a prototype version of the present software was available. In this report, we have since extended this investigation to 10 additional samples, using commercially available software for computer simulation. Our goals for the present paper include the following: (i) to determine how many of these newly-studied samples can be separated adequately ($R_s > 1.0$) by varying T and b while holding other conditions constant; to generalize these results for other samples; (ii) to further verify the reliability of computer-predicted separations when varying T and b and (iii) to investigate some additional applications of computer-assisted method development based on changes in T and b.

4.1. Resolution maps

The software used in this study provides resolution maps which show resolution R_s for the most-overlapped (''critical'') band-pair in the chromatogram as a function of both temperature T and gradient time t_G (t_G defines gradient steepness b when other conditions are held constant; Eq. (2)). Where we refer to ''resolution'', we mean the value of R_s for the critical pair unless stated otherwise. A primary use of these maps is to identify conditions of T and t_G which provide acceptable or maximum resolution for the total sample. It is also possible to assess the value of T and t_G as variables for controlling the selectivity and separation of sub-groups of various sample components. For reference purposes, it is useful to begin our discussion with a resolution map for a homologous series, for which changes in T and t_G do *not* result in useful changes in band spacing [12]. We will use data from Ref. [5] for the separation of the C_3–C_{10} 1-nitroalkanes for this purpose. Fig. 1a shows the nitroalkane resolution map for gradient times of 10–100 min and temperatures of 30–70°C. There is a continuous increase in resolution as gradient time t_G increases, but little change in resolution as temperature T changes (a slight preference for higher temperatures can be seen in Fig. 1a). Resolution for two adjacent bands in gradient elution can be expressed [7,13] as

$$R_s = (1/4)(\alpha - 1)N^{1/2}(k^*/[k^* + 1]) \quad (4)$$

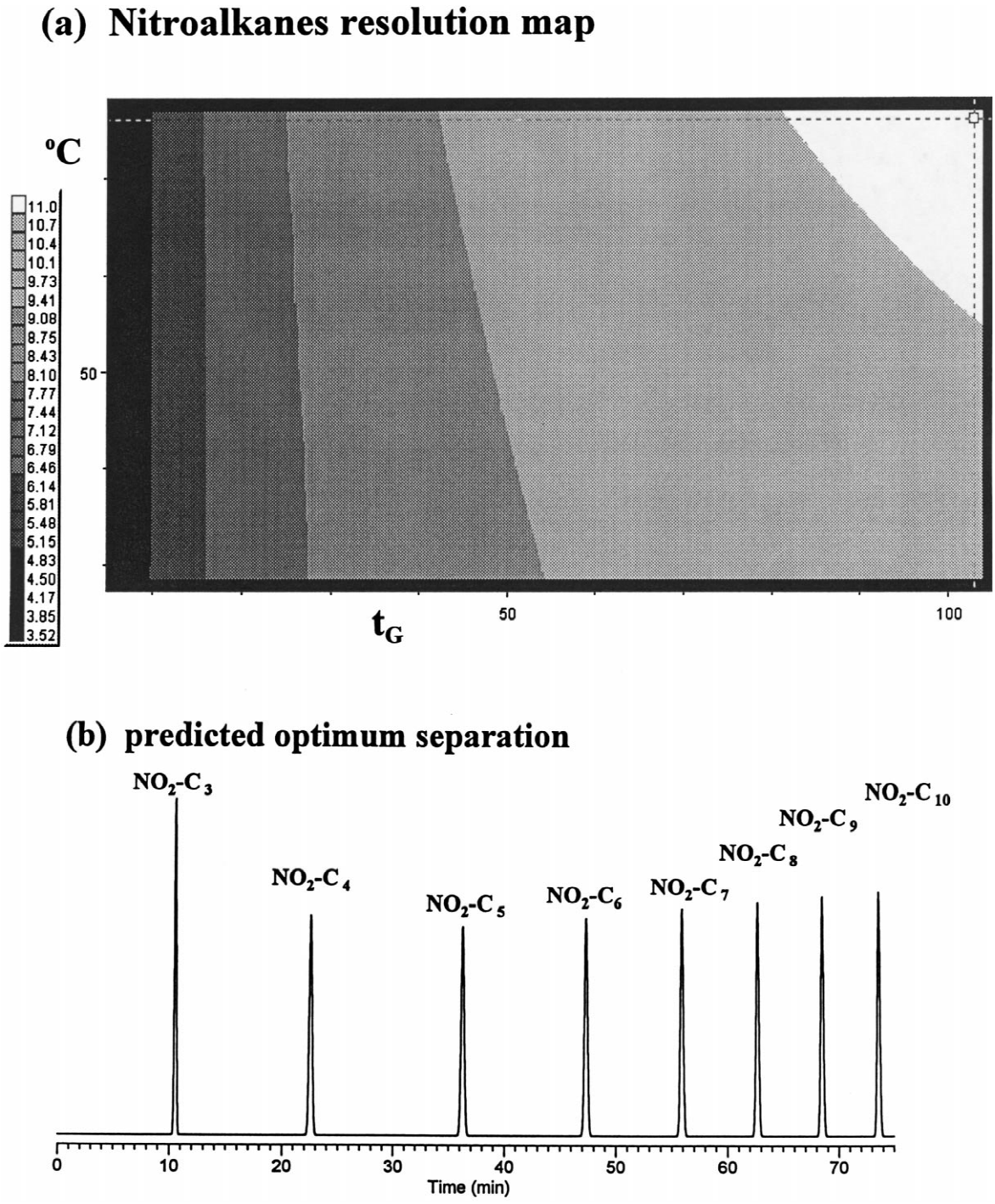


Fig. 1. Optimized separation of eight 1-nitroalkanes. Conditions: 25×0.46 cm C_{18} column; 0–100% ACN in buffer gradient; 2.0 ml/min. (a) Resolution map as a function of temperature and gradient time; (b) predicted chromatogram for 75°C and 104-min gradient (maximum R_s). Cursor (cross-hairs) in (a) indicates conditions for maximum resolution.

where α is the separation factor, N is the column plate number and k^* is an effective retention factor k given by

$$k^* = 1/1.15b \quad (5)$$

The increase in resolution with gradient time in Fig. 1a is largely the result of an increase in k^* (because of larger t_G and b), and according to Eqs. (4) and (5) this effect should level off for larger values of t_G. This is observed in Fig. 1a at 30°C: for t_G = 20, 40, 60, 80 and 100 min, R_s = 7.0, 8.7, 9.5, 10.0 and 10.3. The predicted chromatogram for maximum sample resolution (T = 75°C, 104-min gradient) is shown in Fig. 1b.

4.2. Laboratory F (herbicide sample)

This sample consists of 13 herbicides described in Table 1. Initial experiments for this sample were carried out at 30 and 40°C, in gradient times of 40 and 120 min (for other conditions, see Fig. 2). The resulting resolution map for varying T and t_G is shown in Fig. 2a. Higher resolution occurs for intermediate gradient times (80–100 min) and temperatures <40°C. At lower gradient times, bands 4–6 are critical, while at higher temperatures, bands 9/10 are most overlapped (see Fig. 2b for band numbering). The highest resolution (R_s = 1.56) occurs for T = 34°C and a 90-min gradient (noted by cursor or cross-hairs in Fig. 2a). These and other relevant data for this sample are summarized in Table 2.

Table 1
Components of herbicides sample of laboratory F

(1)	Nicosulfuron
(2)	Thifensulfuron-methyl
(3)	Metsulfuron-methyl
(4)	Chlorsulfuron
(5)	Rimsulfuron
(6)	Azimsulfuron
(7)	Sulfometuron-methyl
(8)	Ethametsulfuron
(9)	Flupyrsulfuron
(10)	Tribenuron-methyl
(11)	Chlorimuron-ethyl
(12)	Bensulfuron-methyl
(13)	Trisulfuron-methyl

Peak assignments are tentative, based on retention in similar HPLC systems.

An experimental run was carried out at 30°C and a time of 80 min, conditions which provide similar resolution (R_s = 1.40) in a shorter run-time. This experimental separation (Fig. 2c) is compared with the computer simulation prediction in Fig. 2b. Experimental vs. predicted retention times show an average deviation of 0.1 min, and retention time differences for adjacent bands (proportional to values of R_s) agree with an average deviation of ±0.01 min (i.e., excellent agreement). A slight fronting of the experimental bands (Fig. 2c) increases their overlap slightly, relative to the predicted chromatogram. The present software can take band asymmetry into account, as illustrated in Fig. 2d for this sample.

4.3. Laboratory A (a synthetic organic mixture)

A mixture of 11 organic compounds (Table 3) has been used by laboratory A as a test sample for studies in temperature-programmed reversed-phase HPLC. Initial experiments for this sample were carried out at 30 and 70°C, in gradient times of 30 and 60 min (for other conditions see Fig. 3). The resulting resolution map for varying T and t_G is shown in Fig. 3a. Higher resolution occurs within a region bounded by $45 < t_G < 55$ min and $30 \leq T < 60$°C, with maximum resolution (R_s = 2.35) for t_G = 45 min and T = 45°C (critical band-pair 7/8). For t_G <40 min, band-pairs 4/5, 6/8 and 7/8 are critical, while for t_G >50 min and T <70°C, bands 6/7 are most overlapped.

The predicted optimized separation (t_G = 45 min and T = 45°C) is shown in Fig. 3b, for comparison with the actual separation in Fig. 3c. Experimental vs. predicted retention times show an average deviation of 0.1 min, and retention time differences for adjacent bands (proportional to values of R_s) agree with an average deviation of ±0.04 min. This is (again) excellent agreement. A slight tailing of the experimental bands increases their overlap slightly, relative to the predicted chromatogram. Correction for band tailing is illustrated in Fig. 3d.

4.4. Laboratory D (acrylate monomers)

This proprietary sample contains 14 monomers

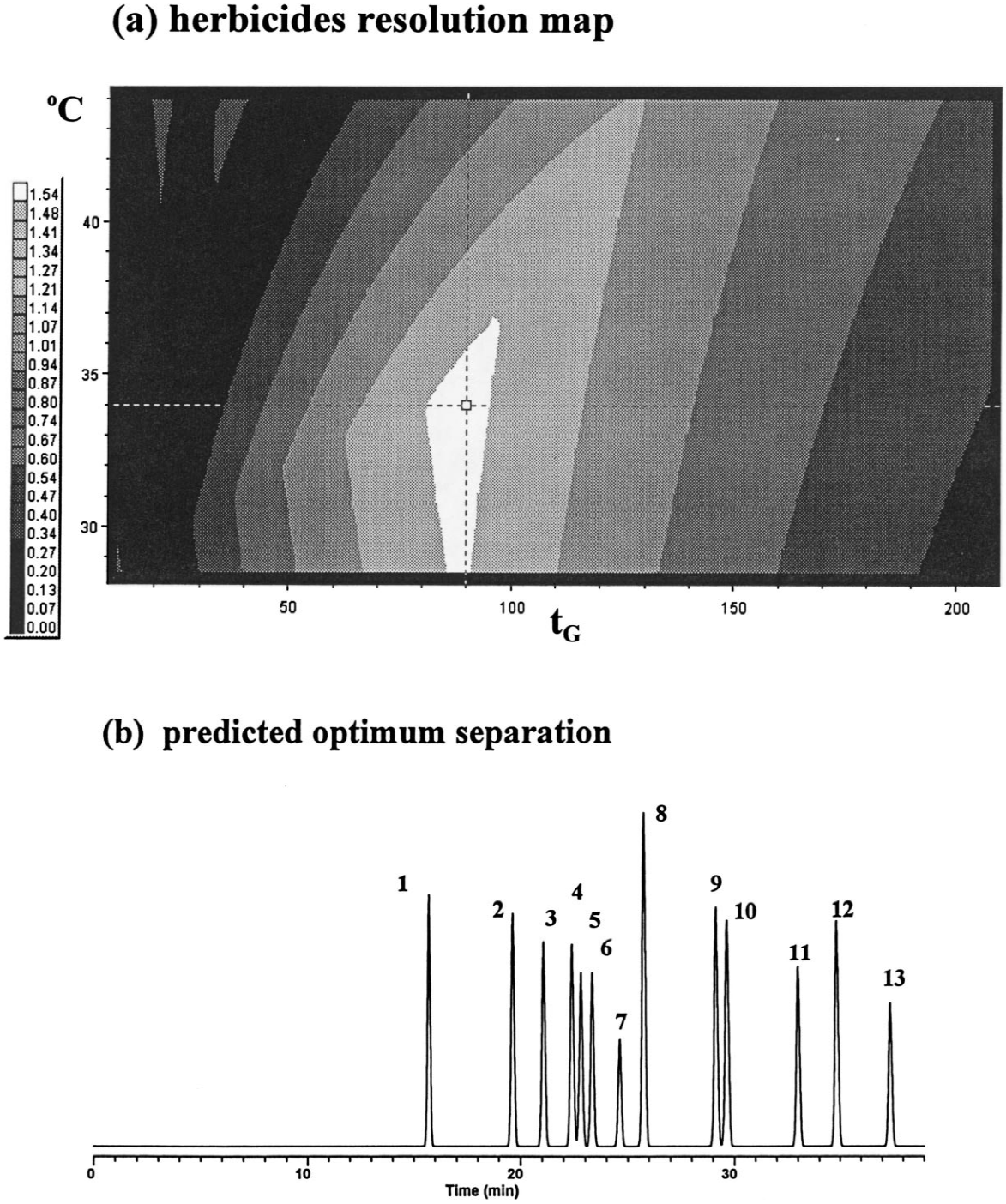
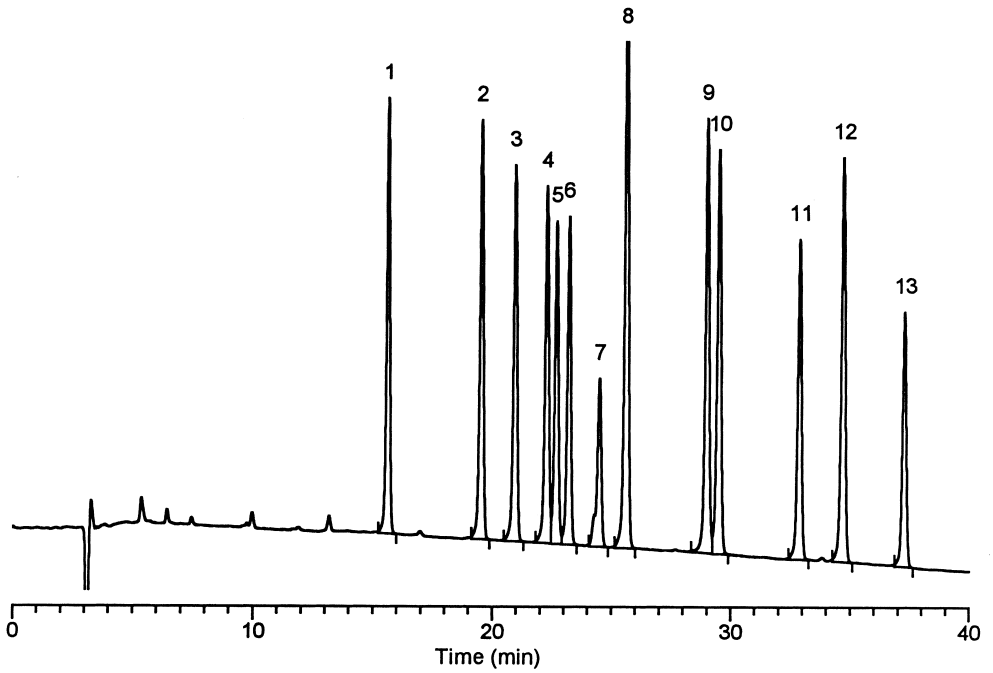


Fig. 2. Optimized separation of 13 herbicides (sample 1 of Table 2). Conditions: 25×0.46 cm C_{18} column, 20–95% acetonitrile in buffer gradients, 1.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 30°C and 80-min gradient; (c) experimental chromatogram for conditions of (b); (d) predicted chromatogram as in (b), corrected for fronting.

(c) actual separation



(d) same as (b), except asymmetry factor = 0.8

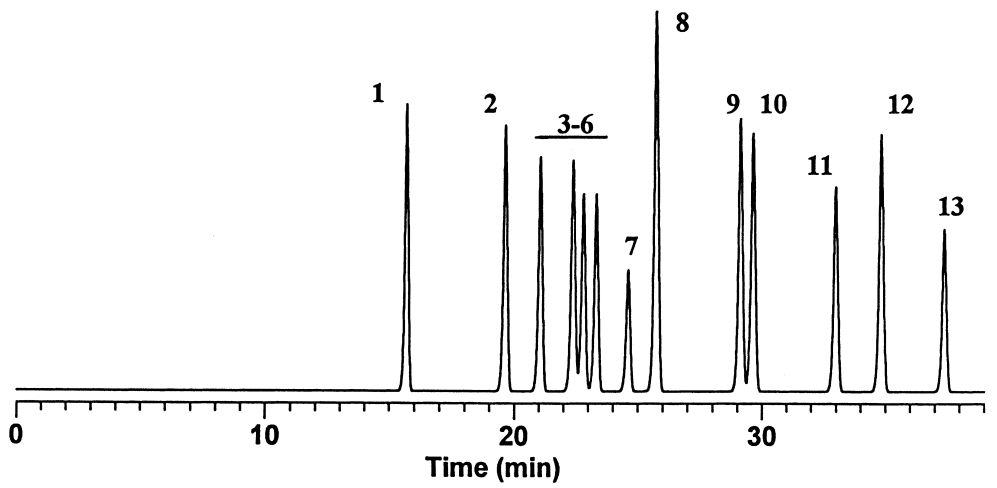


Fig. 2. (*continued*)

Table 2
Summary of results for the separation of samples described in this report

Sample No.	Sample	Conditions (max R_s)		Max R_s	Critical band-pair	n[a]
		t_G (min)	T (°C)			
1	Herbicides	90	34	1.56	9/10	13
2	Synthetic organic mixture	45	45	2.35	7/8	11
3	Acrylate monomers	84	22	1.38	8/9	14
4	Algal pigments	80	57	0.71	16/17	29
5	Fatty acid methyl esters	19	62	0.32	10/11	14
6	Corticosteroids	53	30	1.10	4/6	9
7	Testosterones	42	30	0.20	11/12	17
8	Herbicide impurities	215	38	4.7	7/8	9
9	Pharmaceutical compounds	113	41	6.0	3/4	9
10	Toxicology standards	31	40	0.66	9/10	40
11	Substituted anilines[b]	30	79	3.15	2/3	9
12	Substituted benzoic acids[b]	28	75	1.65	5/7	8
13	LSD derivatives[b]	20	64	0.76	4/5	15
14	Protein digest[b]	206	62	2.96	8/11	20

Maximum resolution (''max R_s'') refers to conditions which provide the largest value of R_s for the most overlapped (''critical'') band-pair.
[a] Number of compounds in sample.
[b] Optimized separations reported in Ref. [3].

(mainly acrylate esters) that vary in alkyl substitution and the number of ester groups in the molecule. Initial experiments with this sample were carried out at 23 and 70°C, with 0–100% acetonitrile in water gradients in times of 20 and 60 min (for other conditions see Fig. 1). The resulting resolution map for varying T and t_G is shown in Fig. 4a. Higher resolution occurs for $T<30$°C and gradient times of 28–31 min and >80 min. For $t_G<40$ min, band-pairs 3/4 and 8/9 are critical, while for $t_G>40$ min, band-pairs 3/4, 5/6 or 8/9 are most overlapped. Maximum resolution ($R_s=1.38$) occurs for $t_G=84$ min and $T=22$°C; Fig. 4b shows the predicted separation for these conditions.

The predicted plate number for the separation of this sample (30-cm, 4-μm particle column) is $N=$ 18 000 (computer simulation). An actual plate number determined by comparing simulated to experimental chromatograms was $N=4000$, which was confirmed by separate isocratic experiments. That is, the column used in these experiments was defective and had apparently lost efficiency with use. The problem of a loss of column plate number can be difficult to recognize when carrying out gradient separations as in the present experiments (even for experienced chromatographers). The ability of the present software to (a) determine an approximate plate number from experimental gradient runs and (b) compare this value with an expected value of N is a useful diagnostic feature.

Table 3
Components of synthetic organic compound mixture of laboratory A

	Thiourea (t_0 marker)	(6)	Naphthalene
(1)	Benzyl alcohol	(7)	1,4-Dichlorobenzene
(2)	Phenothiazine impurity	(8)	Phenothiazine
(3)	Methyl benzoate	(9)	Biphenyl
(4)	Toluene	(10)	1,3,5-Trichlorobenzene
(5)	Benzophenone	(11)	1,2,4,5-Tetrachlorobenzene

4.5. Laboratory E (algal pigments)

This sample contains 29 algal pigments (chlorophylls, carotenoids and related substances) that are listed in Table 4. Initial experiments for this sample were carried out at 50 and 60°C, in gradient times of 17 and 51 min (for other conditions, see Fig. 5). The resulting resolution map for varying T and t_G is shown in Fig. 5a. Highest resolution occurs within two separate regions, both bounded by $t_G>80$ min and $55<T<60$°C. There are numerous bands that become critical at different conditions of T and t_G (Nos. 6–18, 21–23, 27–29), as is expected for this

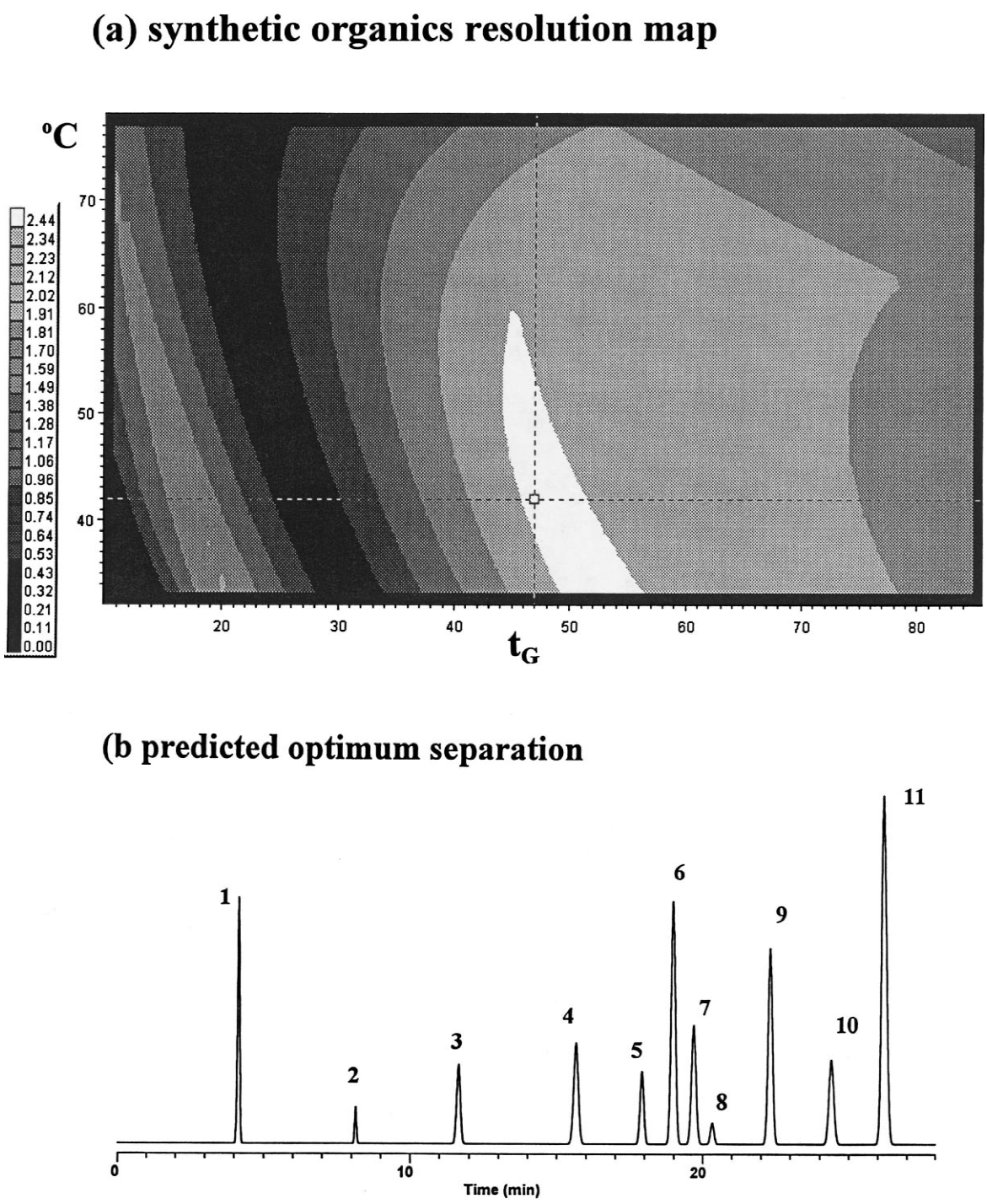
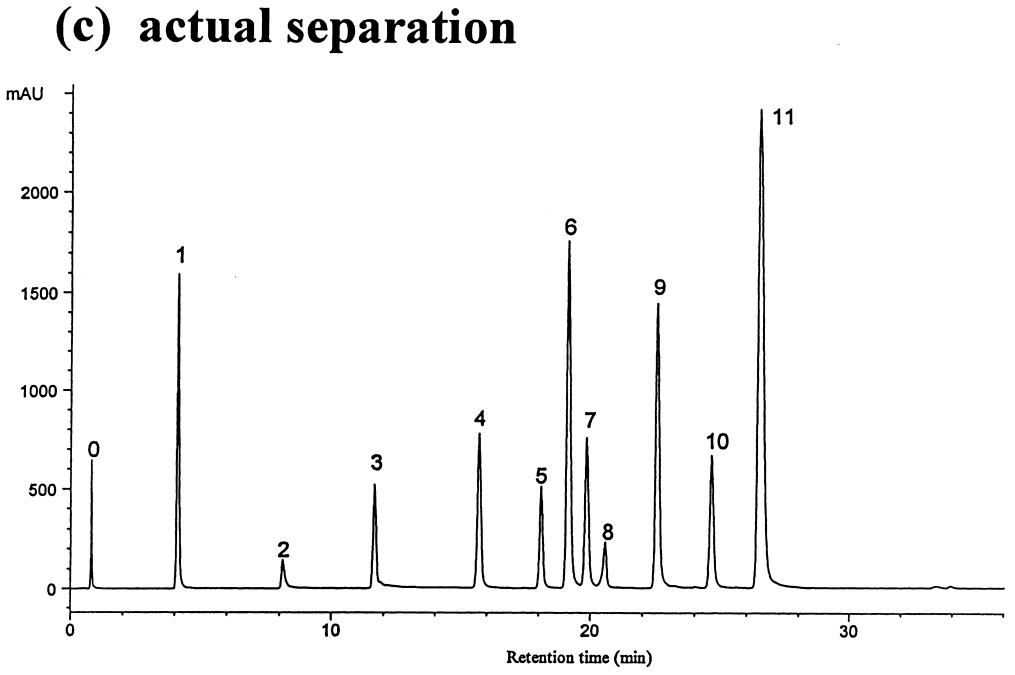


Fig. 3. Optimized separation of 11 synthetic organics (sample 2 of Table 2). Conditions: 12.5×0.30 cm C_{18} column, 10–100% acetonitrile in water, 1.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 45°C and 45-min gradient; (c) experimental chromatogram for conditions of (b); predicted chromatogram as in (b), corrected for tailing. (Continued on p. 10.)



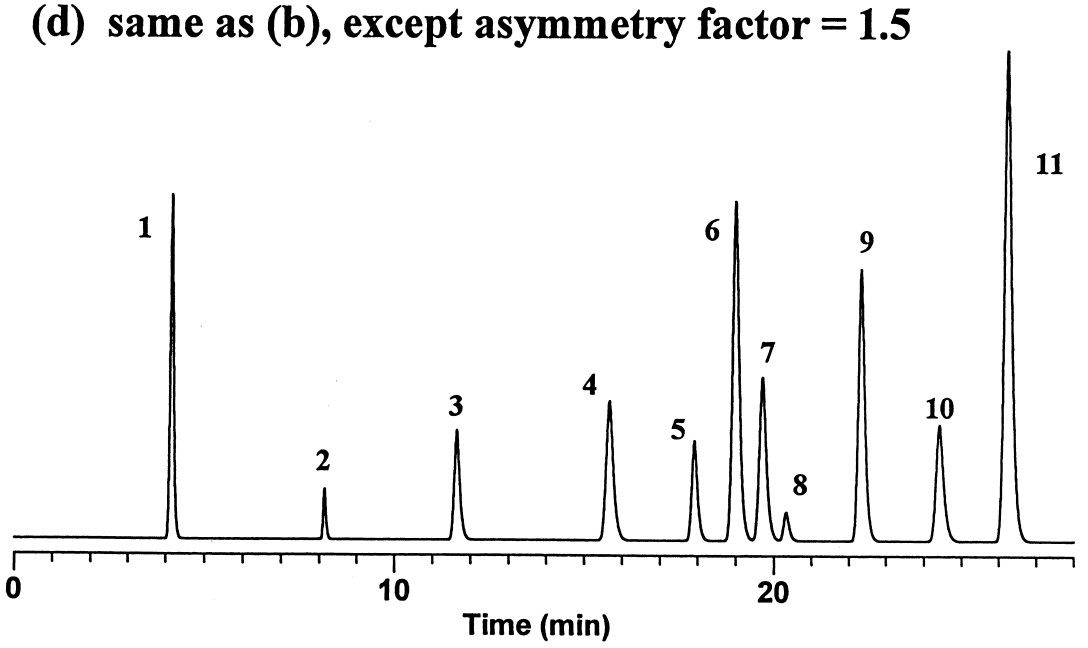


Fig. 3. (*continued*)

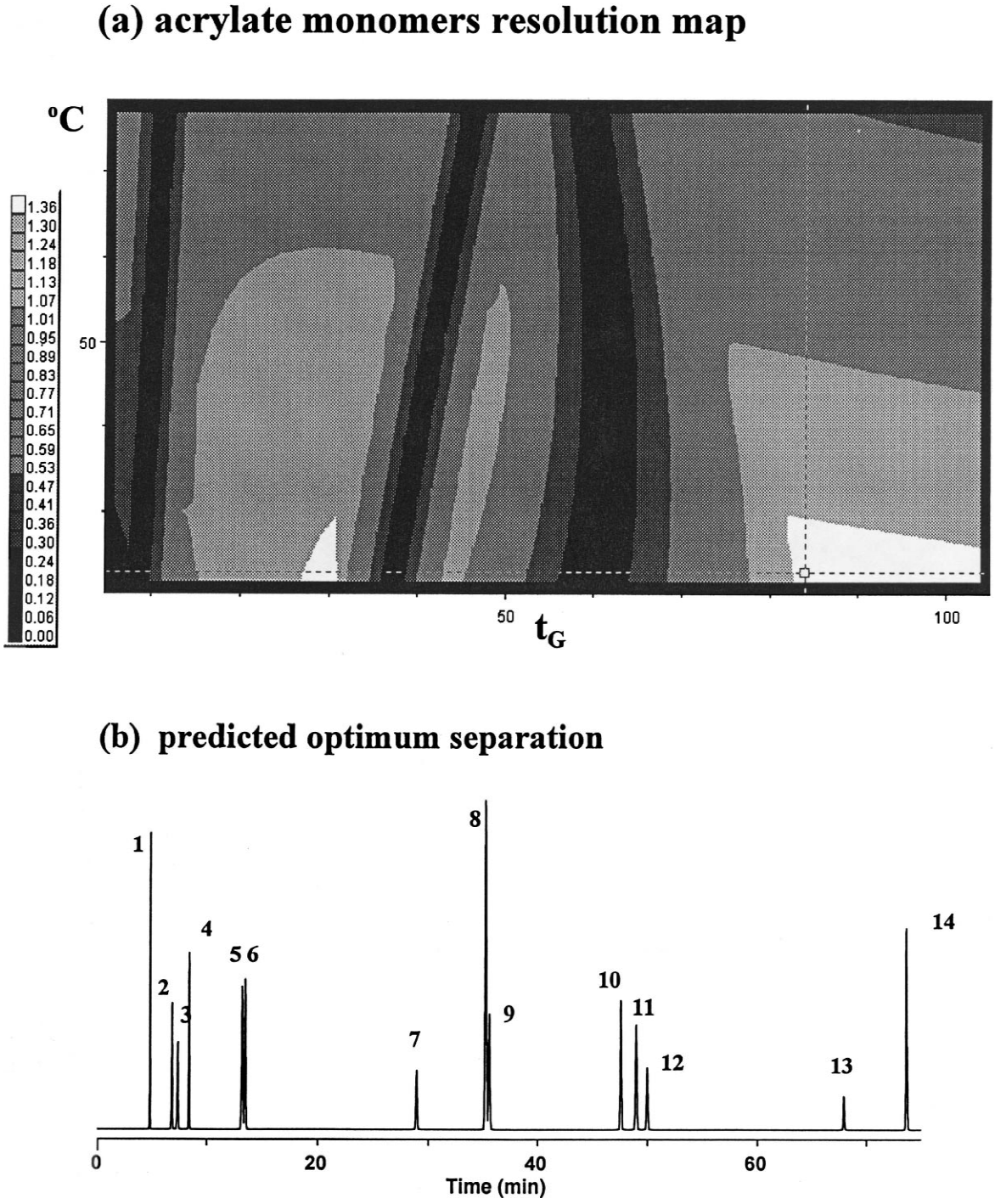


Fig. 4. Optimized separation of 14 acrylate monomers (sample 3 of Table 2). Conditions: 30×0.39 cm C_{18} column, 0–100% acetonitrile in water gradients, 2.0 ml/min; $N=18\,000$ assumed. (a) Resolution map; (b) predicted chromatogram for 22°C and 80-min gradient.

Table 4
Components of algal pigment sample of laboratory E

(1)	Chlorophyll c3	(16)	Chlorophyll b degradant
(2)	Chlorophyll c1 and c2	(17)	Alloxanthin
(3)	Peridinin	(18)	Diatoxanthin
(4)	19′-Butanoyloxyfucoxanthin	(19)	Lutein
(5)	Fucoxanthin	(20)	Zeaxanthin
(6)	19′-Hexanoyloxyfucoxanthin	(21)	Canthaxanthin
(7)	Neoxanthin	(22)	Unknown 2
(8)	Prasinoxanthin	(23)	Chlorophyll b
(9)	Fucoxanthin isomer	(24)	Unknown 3
(10)	Violaxanthin	(25)	Chlorophyll a
(11)	Dinoxanthin	(26)	Chlorophyll a epimer
(12)	Diadinoxanthin	(27)	Phytol c[a]
(13)	Unknown 1	(28)	α-Carotene
(14)	Antheraxanthin	(29)	β-Carotene
(15)	Green algae unknown		

[a] Tentative identification.

very complex sample and resulting crowded chromatogram. Maximum resolution ($R_s = 0.71$) occurs for $t_G = 80$ min and $T = 57$°C; Fig. 5b shows the predicted separation for these conditions.

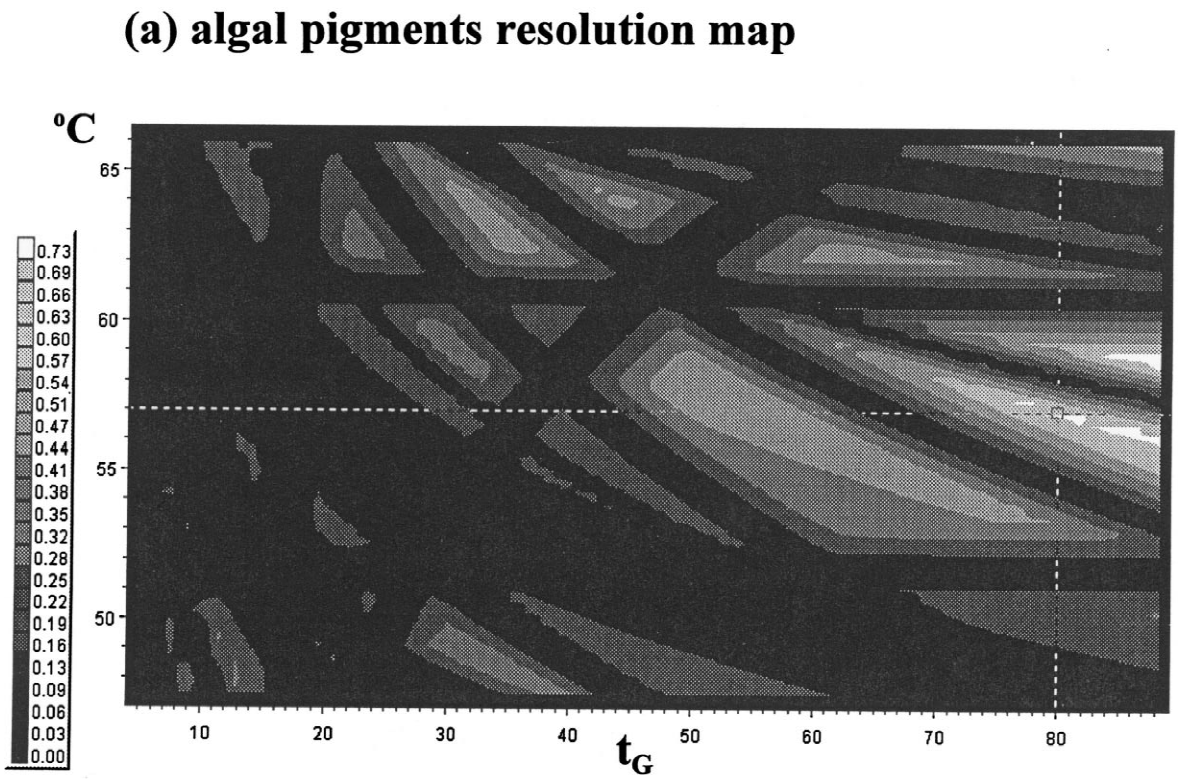
In the present case, it was undesirable to accept a resolution of only $R_s = 0.7$, especially since some of the compounds present in this mixture are rarely found in most samples and can be precluded as possible constituents of other samples. On this basis, it was decided to ignore the overlap of three band-pairs: 8/9, 12/13 and 15/16. The present software allows the user to select peaks whose resolution is unimportant and to recompute a resolution map where the separation of these compounds *from each other* (but not other compounds) is ignored. This revised resolution map is shown in Fig. 5c, where a maximum resolution of $R_s = 1.2$ was possible for all bands except the overlapping pairs 8/9 ($R_s = 0.7$), 12/13 ($R_s = 0.4$) and 15/16 ($R_s = 0.4$). The predicted separation for these conditions (54-min gradient, 55°C) is shown in Fig. 5d, and the actual separation in Fig. 5e. The average error in retention times was −0.06 min, and the average error in retention time differences for adjacent bands was ±0.05 min. This must be considered unexpectedly good agreement, as both input and optimized runs were obtained by injecting subsets of the sample (multiple runs, to avoid confusion over peak tracking [13]), which can result in larger errors in predicted retention times [14].

4.6. Laboratory C (fatty acid methyl esters)

Experimental data suitable for the optimization of T and t_G by computer simulation have been reported [4] for 14 fatty acid methyl esters (described in Table 2 of Ref. [4]). Gradient times of 30 and 90 min were used, with temperatures of 35 and 70°C; other experimental conditions are given in Fig. 6 and Ref. [2]. The resulting resolution map shows a region of maximum R_s in a region bounded by $10 < t_G < 30$ and $55 < T < 65$°C; maximum resolution ($R_s = 0.32$) is obtained for $t_G = 19$ min and $T = 62$°C (Fig. 6b). The resolution of this sample is determined by bands 10 and 11 (methyl esters of oleic and *cis*-7-octadecenoic acids), since the best separation possible for these bands alone is $R_s = 0.32$. If the resolution of these two compounds from each other is ignored (use of a revised resolution map, as in Fig. 5c), a better separation of the remaining bands is possible: a 49-min gradient and 28°C gives a resolution of $R_s = 0.9$ for all band-pairs except 10/11 (Fig. 6c).

4.7. Laboratory B (steroids)

Two samples were selected for optimized separation based on changes in T and t_G (see Table 5): (a) a nine-component mixture of some corticosteroids and their metabolites and (b) a 17-component mixture of testosterone, several monohydroxy metabolites and the 17-keto metabolite (Table 5). The latter



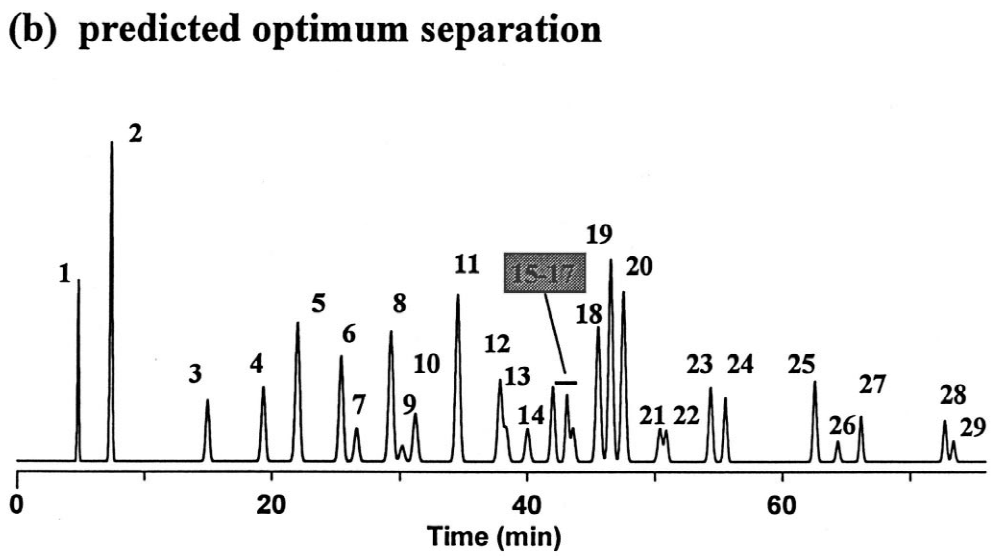
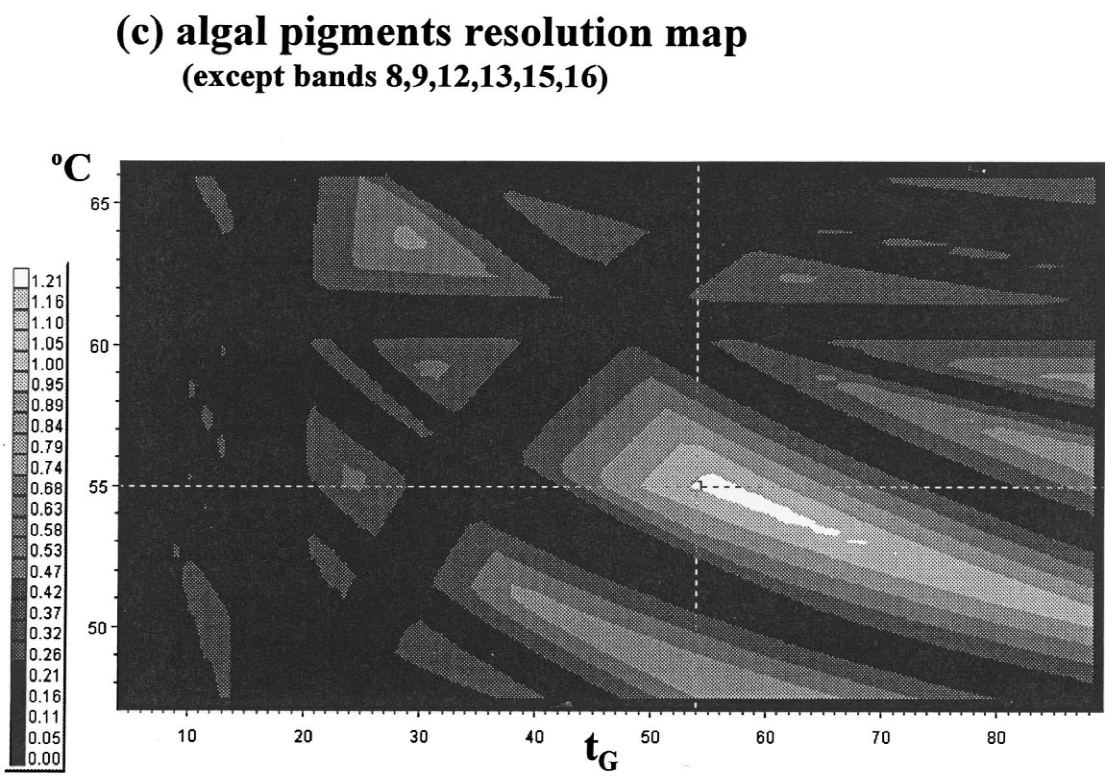


Fig. 5. Optimized separation of 29 algal pigments (sample 4 of Table 2). Conditions: 25×0.32 cm C_{18} column, 70–100% methanol in buffer gradients, 0.65 ml/min. (a) Resolution map; (b) predicted chromatogram for 57°C and 80-min gradient; (c) restricted resolution map, ignoring the separation of peak-pairs 8/9, 12/13 and 15/16 from each other; (d) predicted chromatogram for 55°C and 54-min gradient; (e) experimental chromatogram for conditions of (d), composited from runs on sample sub-sets. (Continued on p. 14.)



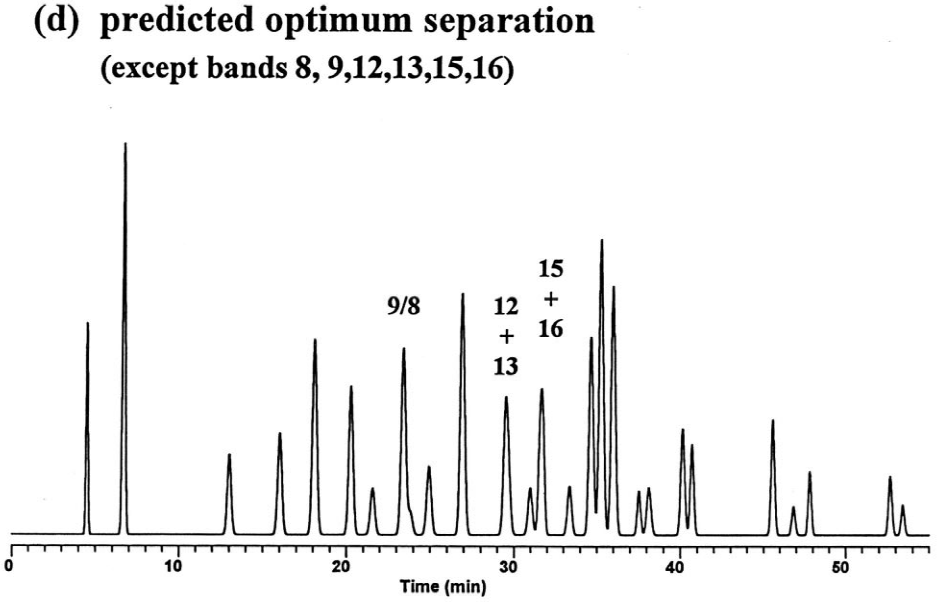


Fig. 5. (*continued*)

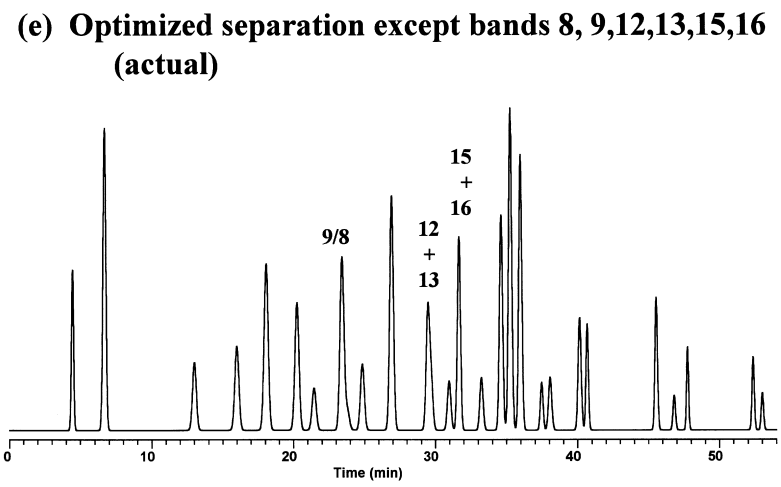


Fig. 5. (*continued*)

sample is of interest in assessing the activities of various cytochrome P-450 enzymes [15,16].

4.7.1. Corticosteroids

For the initial experiments, gradient times of 20 and 60 min were used, with temperatures of 30 and 60°C; other experimental conditions are given in Fig. 7. The resulting resolution map (Fig. 7a) shows a region of maximum R_s bounded by $50<t_G<60$ and $T<35$°C; maximum resolution ($R_s=1.10$) is obtained for $t_G=53$ min and $T=30$°C. Predicted and experimental chromatograms for similar conditions ($t_G=52.5$ min, $T=30$°C; $R_s=1.06$) are shown in Fig. 7b,c. Experimental vs. predicted retention times show an average deviation of 0.03 min, and retention time differences for adjacent bands (proportional to values of R_s) agree with an average deviation of ± 0.04 min (acceptable agreement for HPLC method development).

4.7.2. Testosterones

For the initial experiments, gradient times of 20 and 40 min were used, with temperatures of 30 and 50°C; other experimental conditions are given in Fig. 8. The resulting resolution map shows several regions of maximum R_s (ignore the apparent maximum R_s region at $t_G<9$ min and $T<30$°C, because these conditions are extrapolated excessively). Maximum resolution ($R_s=0.20$) is obtained for $t_G=30$ min and $T=42$°C (Fig. 8b). The separation of Fig. 8a shows two groups of unresolved peaks: Nos. 4–6 and Nos. 11–13. The maximum resolution possible for group 4–6 is $R_s=0.22$; for group 11–13, maximum $R_s=0.48$. It is clear that these six compounds (all are isomers of monohydroxytestosterone) cannot be adequately resolved by any choice of gradient time or temperature.

4.8. Other samples

Data for the separation of three other samples as a function of temperature and gradient time were reported in Ref. [5]. Resolution maps and optimized conditions for their separation will be described.

4.8.1. Herbicide impurities sample

This sample consists of nine structurally-related impurities of a commercial herbicide, as described in Table 4 of Ref. [5]. Initial experiments for this sample were carried out at 39.9 and 57.3°C in times of 10 and 30 min. The resulting resolution map (Fig. 9a) shows maximum resolution for $t_G>20$ min and $T<40$°C. Highest resolution ($R_s=4.7$) occurs for 38°C and a 25-min gradient (Fig. 9b).

4.8.2. Pharmaceuticals sample

This sample consists of nine structurally-dissimilar compounds of pharmaceutical interest, as described in Table 5 of Ref. [5]. Initial experiments for this sample were carried out at 35 and 75°C in times of

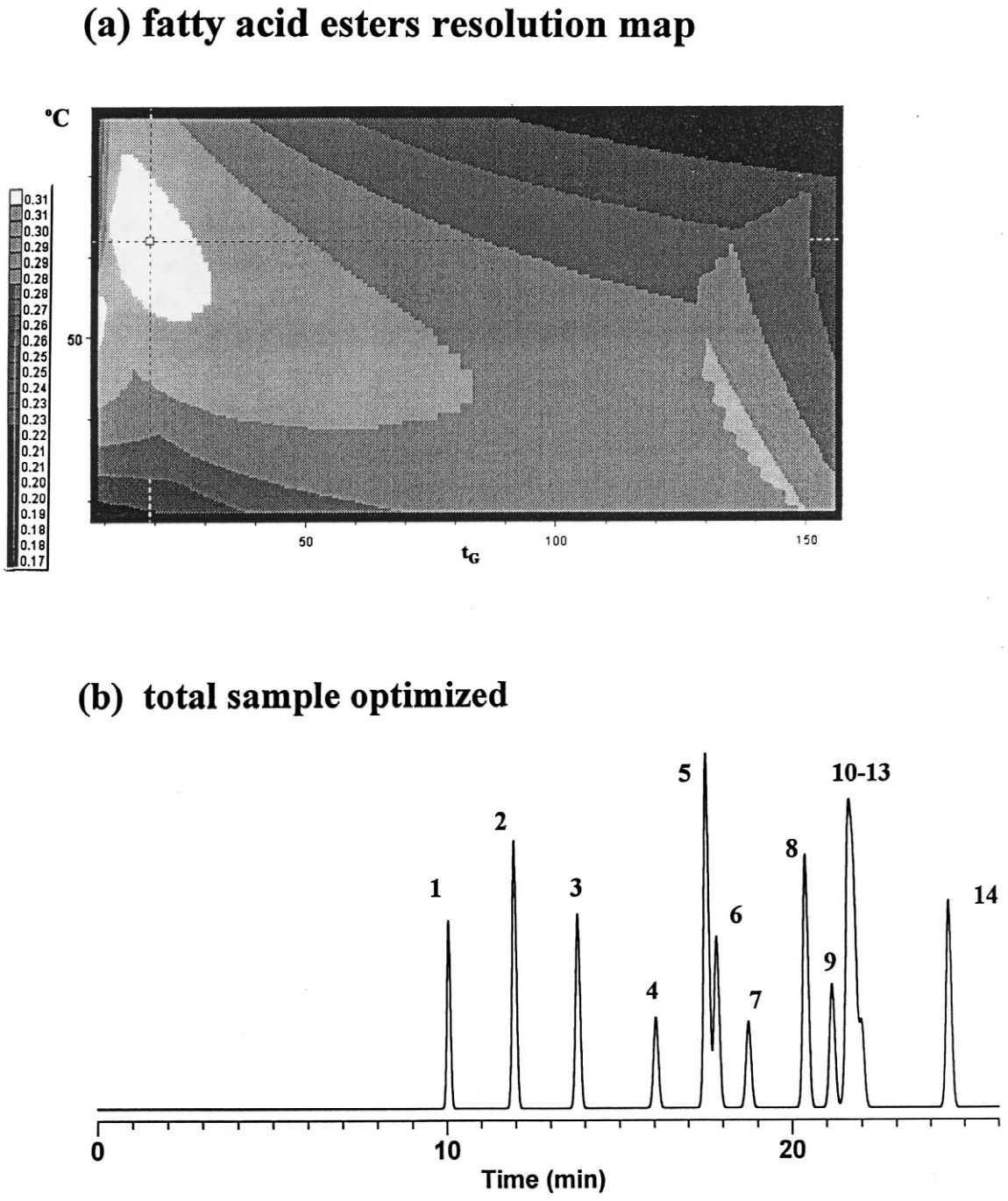


Fig. 6. Optimized separation of 14 fatty acid methyl esters (sample 5 of Table 2). Conditions: 25×0.46 cm Zorbax SB-C_{18} column; 60–100% acetonitrile in water gradient; 1.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 62°C and 19-min gradient; (c) predicted chromatogram for 28°C and a 49-min gradient (ignores overlap of band-pair 10/11).

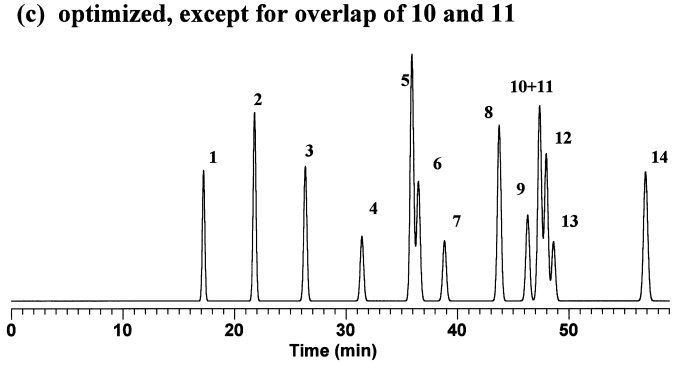


Fig. 6. (*continued*)

30 and 90 min. The resulting resolution map (Fig. 10a) shows maximum resolution for $80<t_G<110$ min and $T>40$°C. Highest resolution ($R_s=6.1$) occurs for 41°C and a 113-min gradient (Fig. 10b).

4.8.3. Toxicology standards

This sample consists of 40 compounds used as standards in a screen for possible toxic substances in body fluids. It is described in Table 3 of Ref. [5], excluding early-eluting basic compounds 1–7 (whose retention cannot be accurately predicted because of pre-elution). Initial experiments for this sample were carried out at 30 and 66.3°C in times of 20 and 60 min. The resulting resolution map is shown in Fig. 11a. The large number of components in this sample result in numerous changes in relative band position as T and t_G are varied, in turn resulting in a large number of local maxima. Highest resolution ($R_s=0.66$) occurs for 40°C and a 31-min gradient (Fig. 11b).

4.9. Previously reported examples of optimizing temperature and gradient time

Four additional samples were reported in Ref. [3] with preliminary resolution maps and optimized conditions for their separation. Revised resolution maps for these four samples are shown in Fig. 12 for

Table 5
Components of steroid samples of laboratory B

Corticosteroids		Testosterones	
(1)	20-Dihydroprednisolone	(1)	7α-Hydroxytestosterone
(2)	20-Dihydroprednisone	(2)	6α-Hydroxytestosterone
(3)	Hydrocortisone	(3)	15α-Hydroxytestosterone
(4)	Prednisone	(4)	15β-Hydroxytestosterone
(5)	Prednisolone	(5)	19-Hydroxytestosterone
(6)	Cortisone	(6)	6β-Hydroxytestosterone
(7)	Betamethasone	(7)	14α-Hydroxytestosterone
(8)	Dexamethasone	(8)	11α-Hydroxytestosterone
(9)	11-Deoxycortisol	(9)	16α-Hydroxytestosterone
		(10)	11α-Hydroxymethyltestosterone
		(11)	2β-Hydroxytestosterone
		(12)	2α-Hydroxytestosterone
		(13)	11β-Hydroxytestosterone
		(14)	18-Hydroxytestosterone
		(15)	β-Hydroxytestosterone
		(16)	Testosterone
		(17)	17-Ketotestosterone

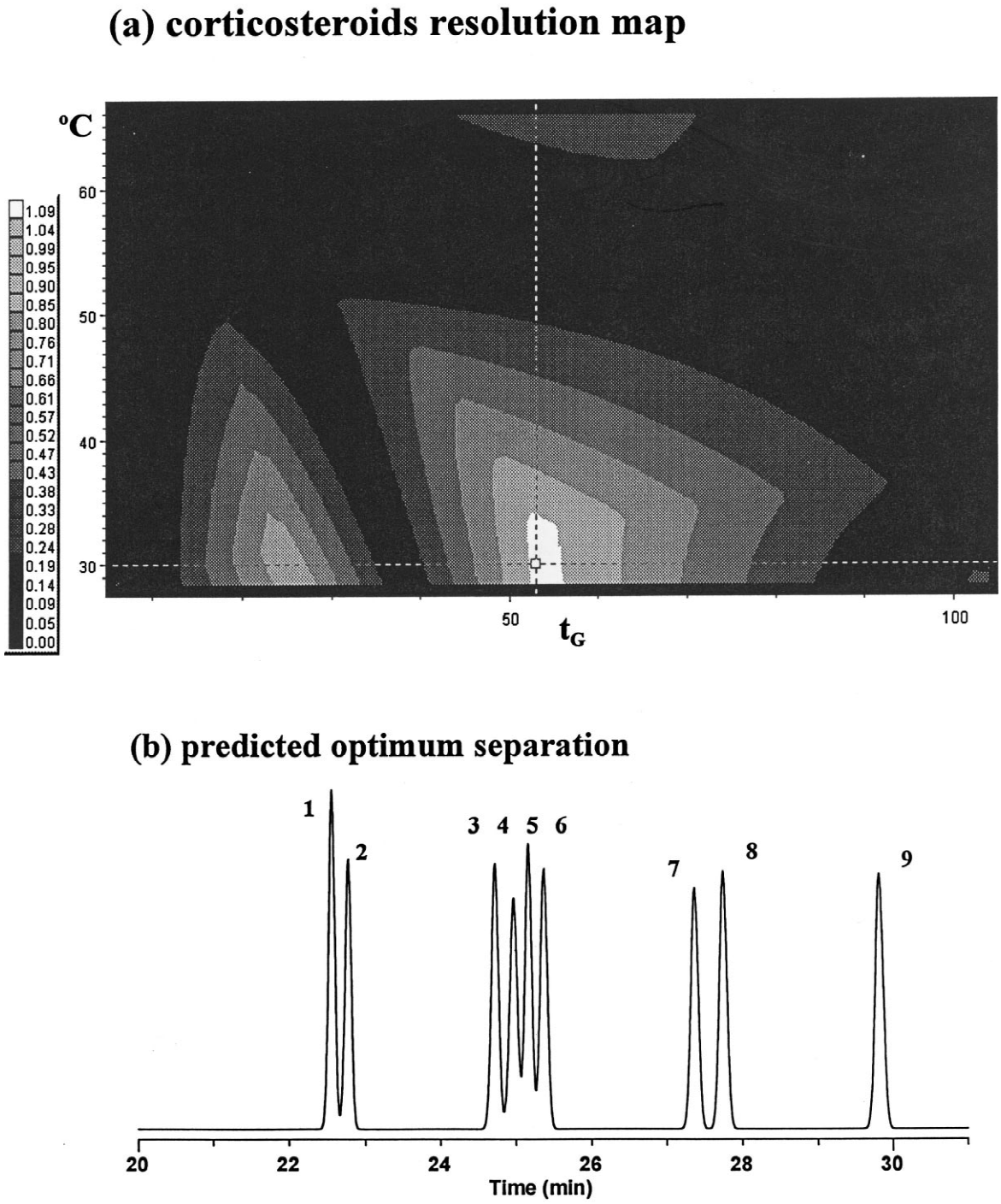


Fig. 7. Optimized separation of nine corticosteroids (sample 6 of Table 2). Conditions: 25×0.46 cm C_8 column; 0–80% acetonitrile in buffer gradient; 2.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 30°C and 52.5-min gradient; (c) experimental chromatogram for conditions of (b).

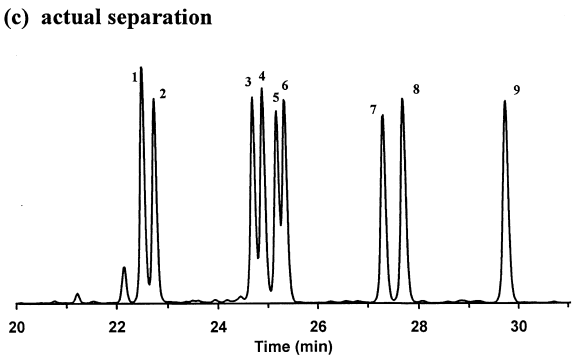


Fig. 7. (*continued*)

further discussion here. These samples include (a) a nine-component substituted anilines sample, (b) an eight-component substituted benzoic acids sample, (c) a 15-component mixture of LSD derivatives and (d) a tryptic digest of recombinant human growth hormone that contains 20 major peptides.

5. Discussion

We have examined the optimization of 10 new samples by means of changes in temperature and gradient steepness. These results have been supplemented with similar data reported previously for four additional samples [3]. These 14 samples, whose separation is summarized in Table 2, include three mixtures of synthetic organic compounds and 11 samples of ''real'' interest to the chromatographers involved. Thus, conclusions that might be drawn from these separations should be more broadly applicable than past studies of a similar nature where only one or a few samples have been used to evaluate a resolution–optimization procedure for reversed-phase HPLC (RPLC).

5.1. How effective are temperature and gradient time?

5.1.1. Separation of the sample

Several of the samples of Table 2 were expected to be more difficult to separate by virtue of component similarity (i.e., Nos. 3, 5, 7, 8, 11), or because they contained a large number n of components ($n \geq 15$ compounds; i.e., Nos. 4, 7, 11, 12, 15). Of the 14 samples summarized in Table 2, it was possible to separate nine of these samples with a resolution $R_s > 1.0$ and only two samples (Nos. 5 and 7) had maximum $R_s < 0.6$. A previous study of sample 5 (fatty acid methyl esters) has shown that its band spacing does not change much as T and t_G are varied, compared to other samples [2]. This limited change in selectivity with T or t_G can be attributed to the fact that all but one of the components of this sample have only one (and the same) polar group in the molecule (methyl ester, $-CO_2CH_3$), since a later study [4] has shown a high correlation between band-spacing changes with T and t_G and *differences* in polar substituents among the components of a sample. Likewise, sample 7 contains 14 isomers of hydroxytestosterone, and the hard-to-separate compounds in this sample are in each case isomers; i.e., compounds with no difference in either polar or non-polar substitution.

Considering the challenging nature of the present samples, it seems reasonable to anticipate an acceptable success rate for the application of this four-experiment method development approach to other samples requiring HPLC separation. As discussed in the following paper [17], further optimization of the gradient (change of initial % B) combined with changes in various experimental conditions (four to eight additional experiments) can significantly increase our chances for success (leaving only samples 4 and 7 with a resolution $R_s < 0.9$). Samples as complex as Nos. 4 and 10 (29–40 components) were not expected to be separable with $R_s > 1$ in a single HPLC separation, because of crowding of the chromatogram and a limited peak capacity of the system. However, the present software provides an alternative solution to this problem by facilitating the development of assay procedures which use two or more HPLC runs (each based on different temperatures and gradient times). In this way, all the components of the sample can be separated in one run or another. The various separated analytes can then be quantitated individually and results for the different runs composited into a report for the total sample. A full description of this multi-run approach to HPLC separation and analysis is in preparation.

5.1.2. ''Excessive'' selectivity effects

The main emphasis in our investigation of the

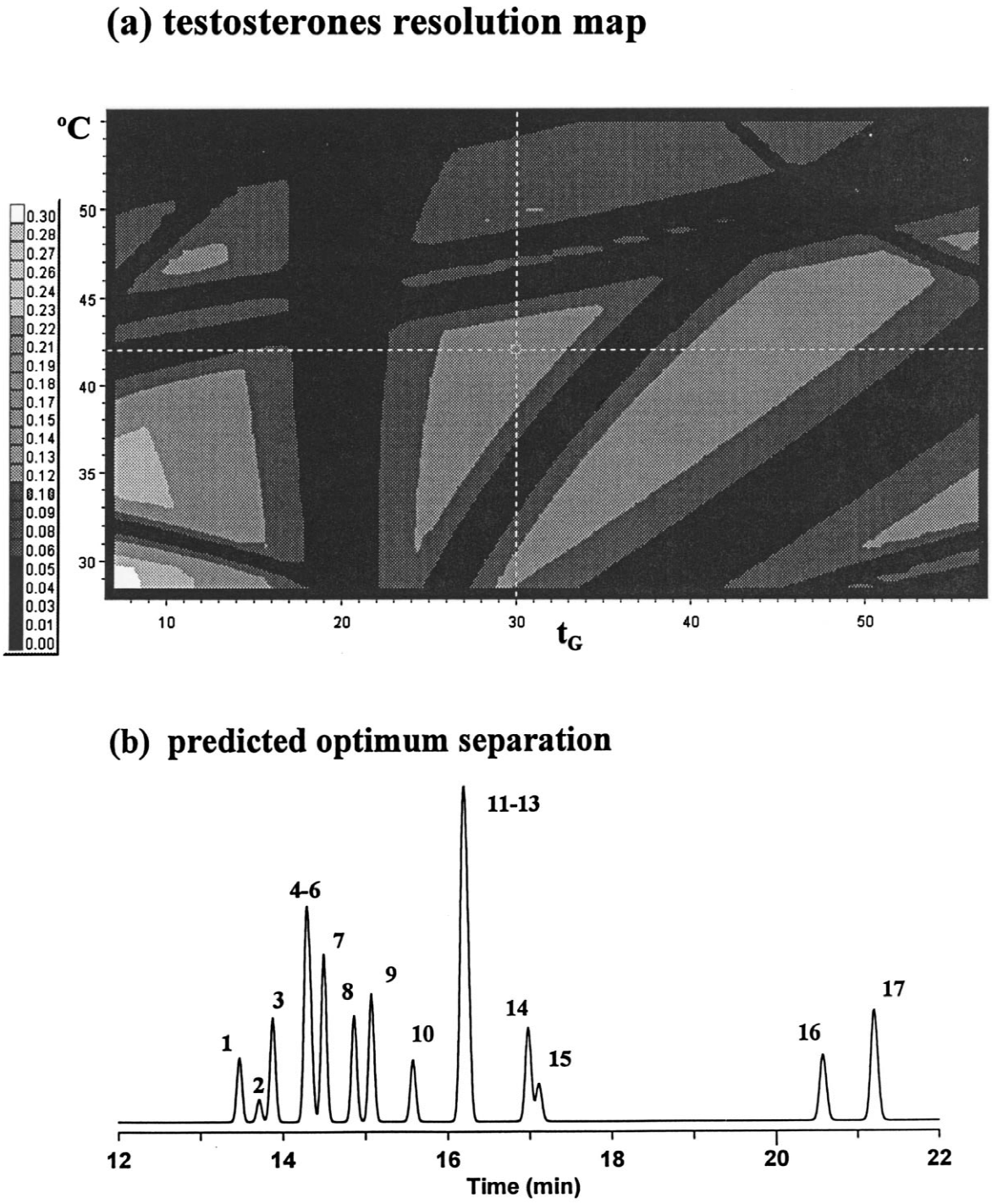


Fig. 8. Optimized separation of 17 testosterone derivatives (sample 7 of Table 2). Conditions: 25×0.46 cm C_8 column; 0–80% acetonitrile in buffer gradient; 2.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 42°C and 30-min gradient.

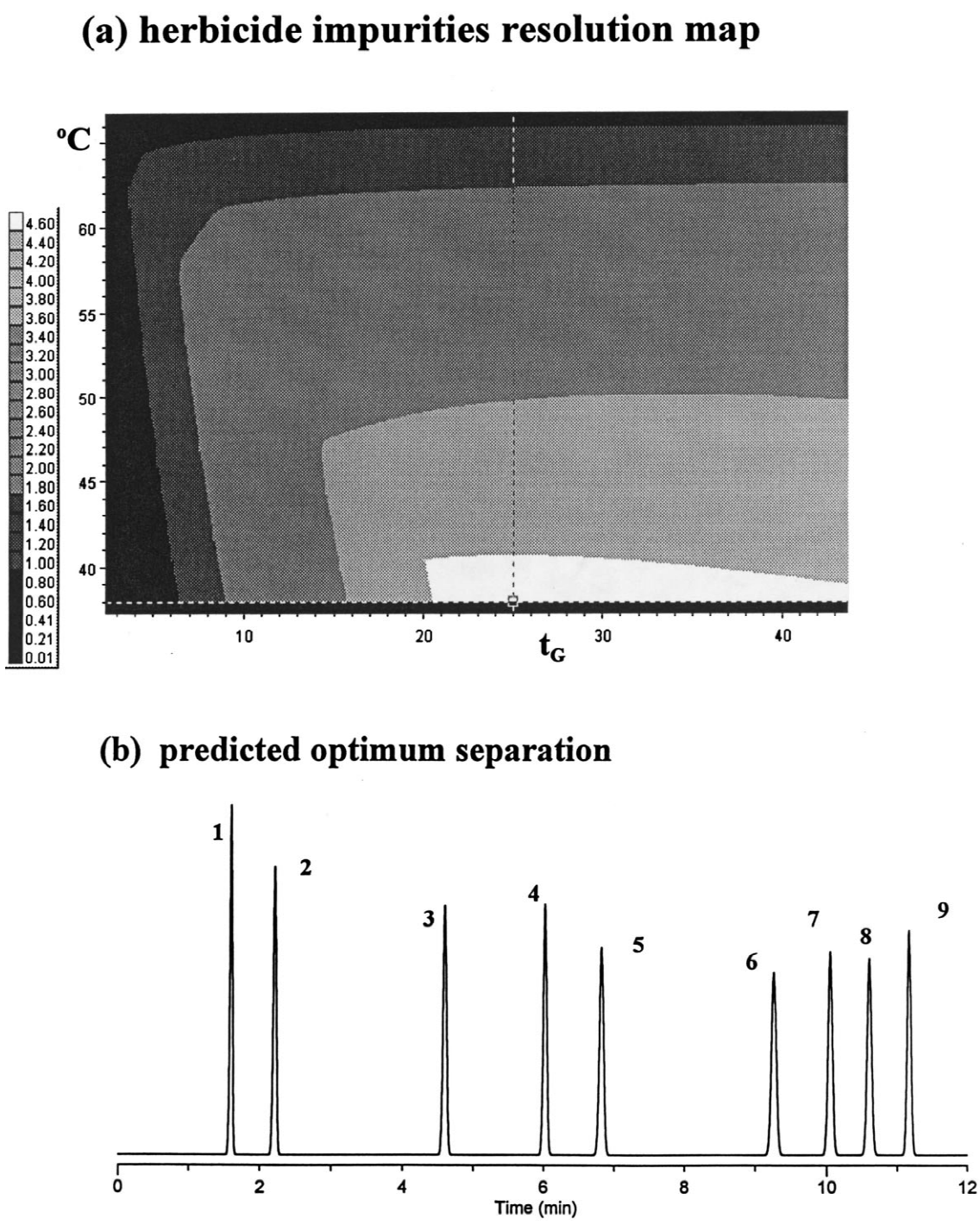


Fig. 9. Optimized separation of 9 herbicide impurities (sample 8 of Table 2). Conditions: 7.5×0.46 cm C_{18} column; 5–95% ACN in buffer gradient; 2.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 38°C and 25-min gradient.

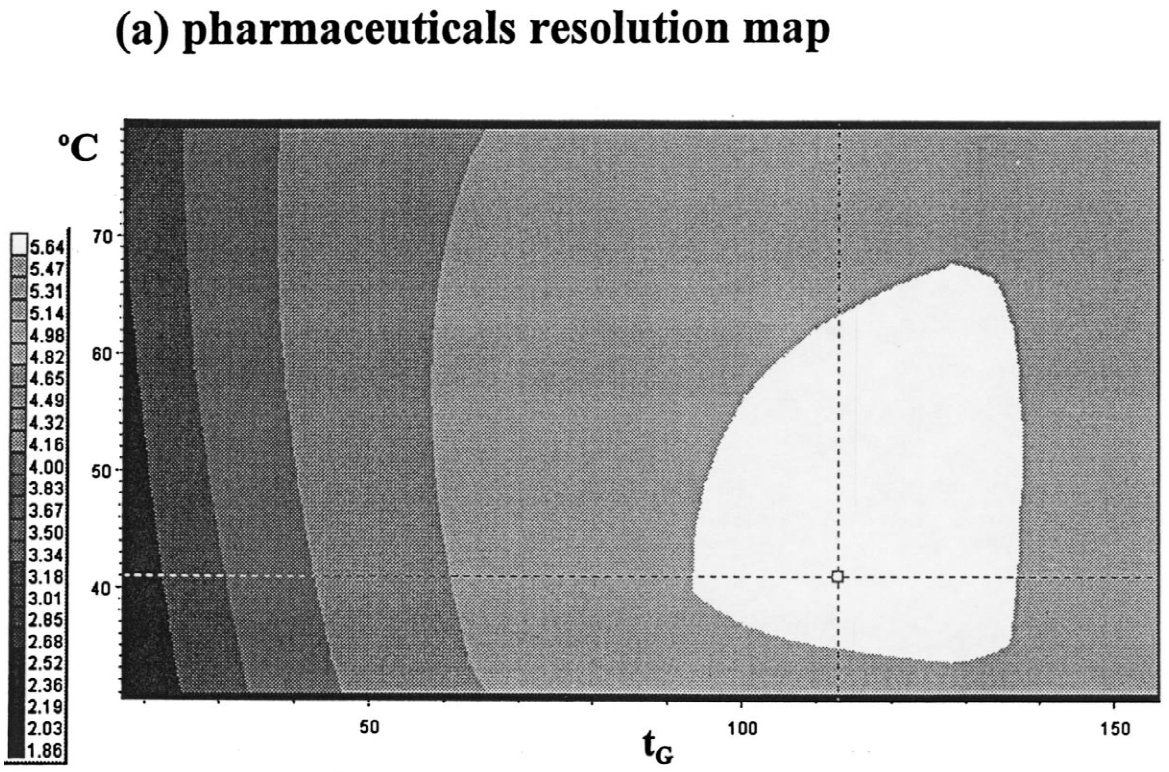


Fig. 10. Optimized separation of nine pharmaceuticals (sample 9 of Table 2). Conditions: 25×0.40 cm C_{18} column; 20–100% ACN in buffer gradient; 1.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 41°C and 113 min.

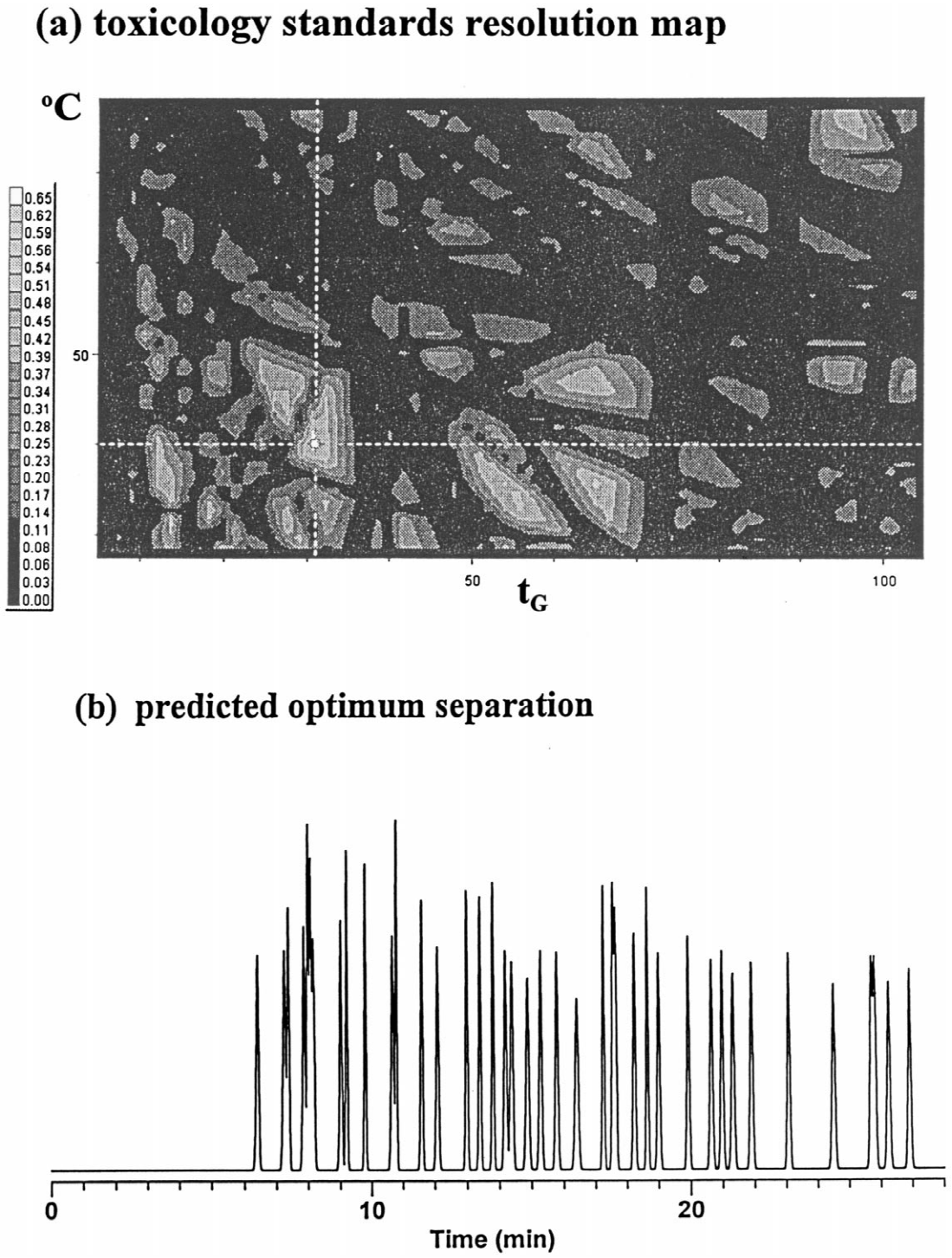


Fig. 11. Optimized separation of 40 toxicology standards (sample 10 of Table 2). Conditions: 25×0.46 cm C_{18} column; 0–100% ACN in buffer gradient; 2.0 ml/min. (a) Resolution map; (b) predicted chromatogram for 40°C and 31-min gradient.

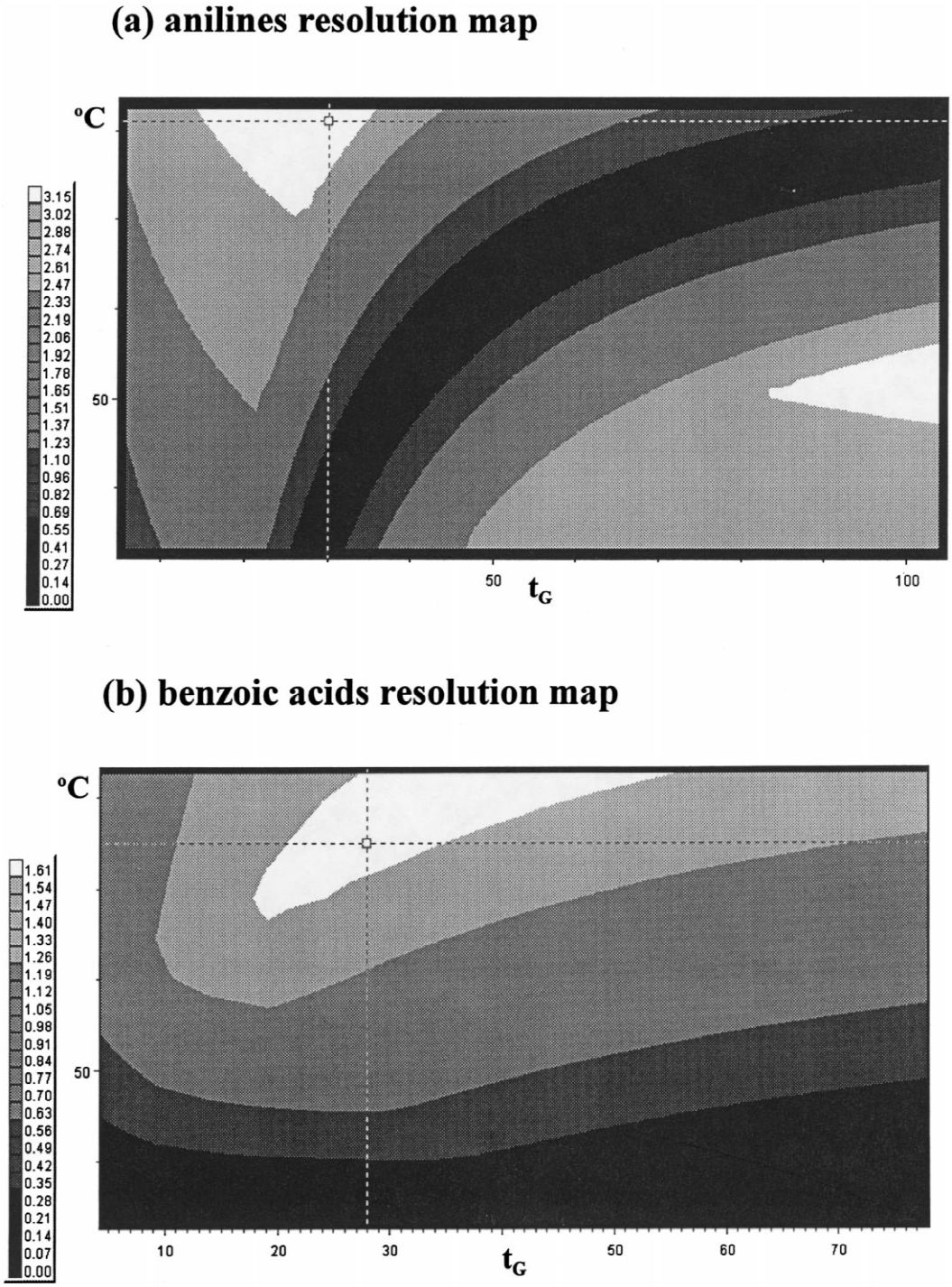
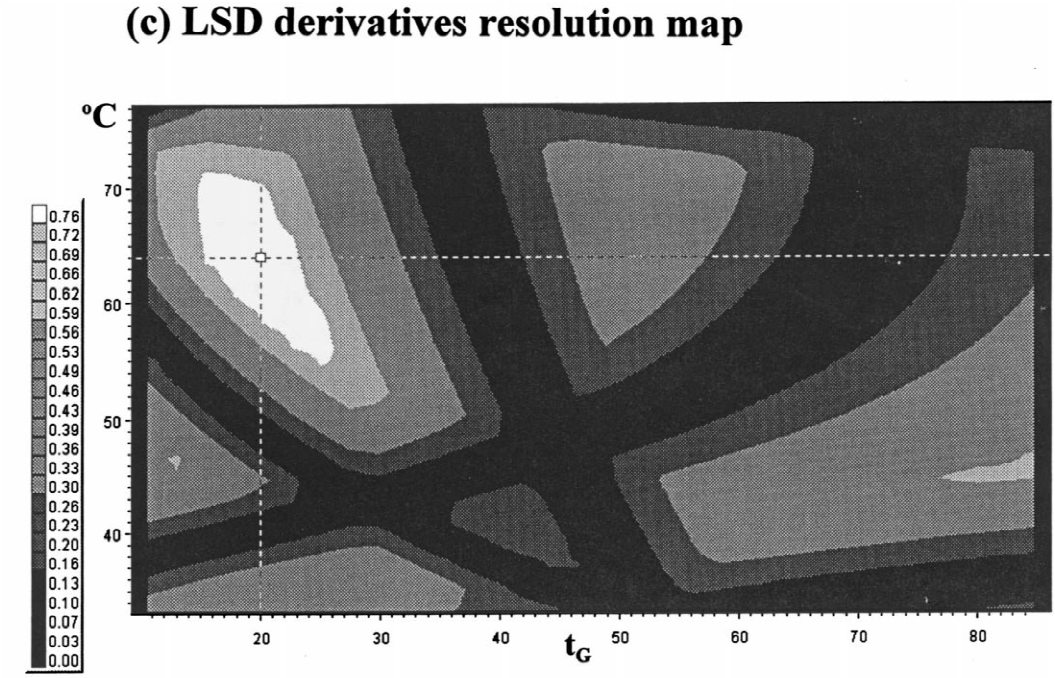


Fig. 12. Resolution maps for samples 11–14 of Table 2. (a) Nine substituted anilines; conditions: 15×0.46 cm C_{18} column; 5–65% ACN in buffer gradient; 1.0 ml/min; (b) eight substituted benzoic acids; conditions: 5–50% ACN in buffer gradient; 1.0 ml/min; (c) 15 LSD derivatives; conditions: 25×0.40 cm C_{18} column; 20–100% ACN in buffer gradient; 1.0 ml/min; (d) 20 peptides from the tryptic digest of recombinant human growth hormone; conditions: 15×0.46 cm C_8 column; 0–60% ACN in buffer gradient; 1.0 ml/min.



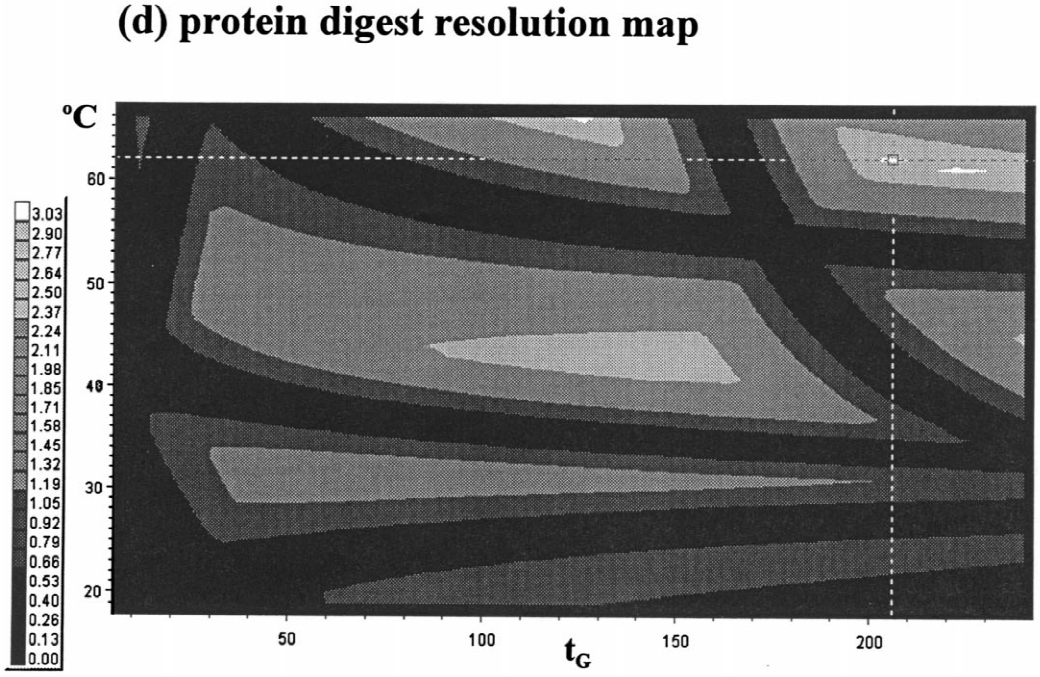


Fig. 12. (*continued*)

effects of temperature and gradient time on band spacing has been placed on the ability of these variables to achieve adequate changes in selectivity and thereby achieve the separation of overlapping bands. Occasionally, large changes in relative separation can result for a pair of adjacent bands as a result of small changes in T and t_G. Examples of this kind can lead to unexpected problems in the present method development procedure, based on optimizing T and t_G. An example of this kind is illustrated in Fig. 13, for compounds 11 (sulfmethanzine) and 12 (brucine) from the chromatogram of Fig. 11b (bands 11 and 12 elute at 10.7 min). The resolution map shown in Fig. 13 is for just these two compounds, whose resolution is $R_s = 8.0$ for the upper-left corner of the map, and $R_s = 18.1$ for the lower-right corner. Since there is a band-reversal between these two chromatograms, the corresponding *change* in resolution is 26 units. This represents a remarkable change in selectivity, regardless of the separation condition being varied.

Two problems can result from samples that include compounds that behave in similar fashion as the example of Fig. 13. First, once conditions have been achieved for the separation of the *total* sample, the resolution of two compounds as in Fig. 13 may be only marginal. This is the case in the separation of the toxicology standards sample in Fig. 11b, where $R_s = 1.02$ for bands 11 and 12 (doublet eluting at 10.7 min). If conditions are changed only minimally (41°C and 30 min-gradient vs. original 40°C and 31 min in Fig. 11b), the resolution of this band-pair decreases to $R_s = 0.56$; i.e., a decrease in resolution by half. That is, the method of Fig. 11b is very likely to suffer from lack of ruggedness and transferability to another HPLC instrument.

A second problem concerns the accuracy of predictions from computer simulation. Just as small changes in conditions or equipment may result in a large decrease in resolution of two bands such as 11 and 12 in Fig. 13, small errors in the computer calculation procedure can translate into equivalent errors in temperature and gradient time. This can be inferred from data in Ref. [5], which show slight curvature of plots of retention time vs. temperature, whereas the present software assumes a linear relationship (Eq. (3). The result will be predictions where the assumed values of T and t_G differ slightly from actual values. For band-pairs such as 11 and 12 in Fig. 13, significant errors in predicted resolution may then result. When problems of this kind are observed (differences in experimental and predicted chromatograms for optimized conditions), an effective response is to use computer simulation to redetermine values of T and t_G which match the initially optimized experimental separation, following which corrected values of T and t_G for maximum resolution can be inferred and tested experimentally.

5.1.3. Effect of temperature and gradient time ranges

In most of the examples of Table 2, the separation conditions for the four initial experimental runs fall short of exploring maximum changes in T or t_G (e.g., 30–90°C, 10-fold change in t_G). The use of a wider range in these conditions should increase the likelihood of further increases in sample resolution, at least for some of these samples. The examples of Figs. 2–12 allow maximum resolution to be mapped as a function of the range in temperature and gradient time used in the initial four experiments for computer simulation, as summarized in Table 6. As expected, maximum resolution increases with an increasing range in T and t_G, and a near-doubling of maximum resolution can be expected when the temperature range is increased from 10 to 60°C and the gradient time range is increased from 1.5-fold to about 10-fold. The data of Table 6 are averages for all 14 samples, and maximum resolution for individual samples shows a varied dependence on the range of T and t_G values. When the separation of a critical band-pair is unresponsive to changes in T and t_G, then sample resolution will also be unresponsive to an increase in the range of T and t_G that is explored. This was the case for band-pair 10/11 in the fatty acid ester sample (No. 5), band-pair 11/12 in the testosterone sample (No. 7) and band-pair 4/5 in the LSD sample (No. 14). As a result, the separation of these samples cannot be improved by varying T and t_G over wider limits.

The choice of initial temperature and gradient time also affects the accuracy of computer simulation for other conditions of T and t_G. A general discussion of predictive accuracy vs. chromatographic conditions is given in [18] for the case of % B or gradient time as variable. Predictions for interpolated conditions

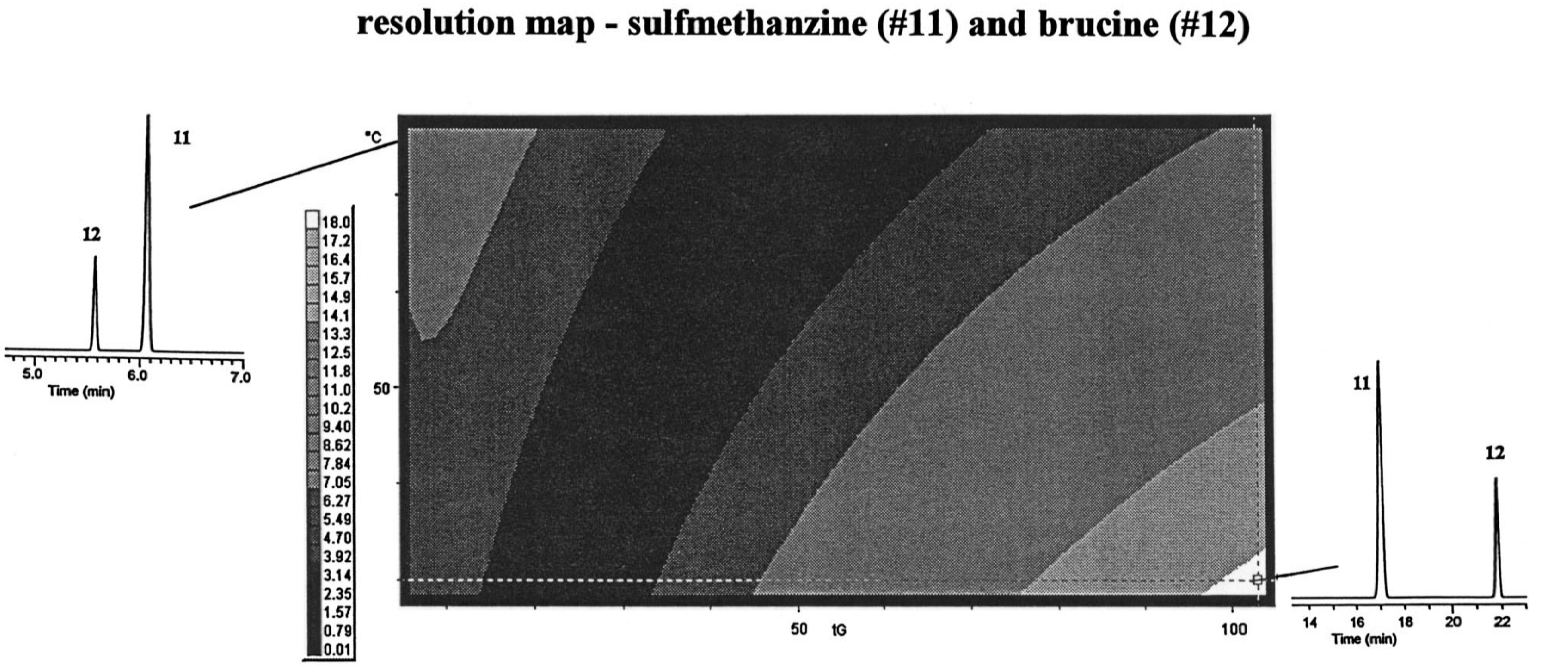


Fig. 13. Resolution of sulfmethanzine (11) and brucine (12) as a function of temperature and gradient time. See Section 5.1.2 for details.

Table 6
Average effect on maximum resolution of the range in temperature and gradient time used for computer simulation

Range		Maximum R_s (relative)
T (°C)	t_G (-fold)	
10	1.5	0.7
20	2.3	0.8
30	3.4	0.9
40	5.1	(1.0)
50	7.8	1.1
60	11.4	1.2

(intermediate between those of the four initial experimental runs [''input runs'']) are generally more reliable than those for extrapolated conditions, except when the difference in the two values of T or t_G become large. Differences in t_G for the input runs can be as large as 4-fold with no adverse effect on the accuracy of predictions for intermediate (''interpolated'') values of t_G. Similarly, extrapolation by a factor of as much as 0.5 $\log(t_{G2}/t_{G1})$ is also reliable. A previous study [5] suggests that a temperature range of at least 35°C for the input runs is allowable, with acceptably accurate predictions for intermediate temperatures. Certain sample/column combinations can result in larger errors [5], and this possibility is currently under further study.

5.2. Optimizing run-time and resolution

The preceding discussion has emphasized maximum sample resolution, but chromatographers are often equally interested in short run-times. The resolution map allows an easy trade of resolution for run-time, when the maximum resolution is greater than necessary (e.g., $R_s > 2$). For example, consider the herbicide impurities sample of Fig. 9. Maximum resolution ($R_s = 4.7$) occurs for a gradient time of 25 min, but much shorter gradients can still provide baseline resolution; e.g., a 7.5-min gradient at 57°C, for which $R_s = 2.0$. In other cases, there is only a marginal loss of resolution from the selection of a shorter gradient time; e.g., acrylate monomers sample of Fig. 4, where gradient time can be shortened from 84 to 29 min, with a negligible loss in resolution ($R_s = 1.33$ for 29 min, vs. 1.38 for 84 min).

5.3. Isocratic separation

Some of the samples of Table 2 can be separated isocratically, based on the rule that isocratic elution is feasible whenever the fraction of a 0–100% B gradient chromatogram that encompasses all peaks of interest is less than 25% [13]. The four gradient experiments used for computer simulation can also be used to predict isocratic separation as a function of T and % B. Because of the similarity of isocratic and gradient elution [11], especially for the separation of peaks within a narrow retention range, the optimum temperature for both isocratic and gradient elution will generally be similar. Two gradient runs at this optimum temperature can be simulated, then used for subsequent predictions of isocratic separation [13] at the same temperature.

As an example, the corticosteroids sample (No. 6) can be separated isocratically. The optimum temperature for the gradient separation of the corticosteroids sample is 30°C; two gradient runs (20 and 60 min) were predicted for this temperature, followed by re-entry of these data into the DryLab program. Based on these simulated inputs, the isocratic resolution map of Fig. 14a became available. Maximum resolution ($R_s = 1.29$) is obtained for a mobile phase of 41% B (acetonitrile in water), as indicated by the cursor in Fig. 14a. The resulting separation is shown in Fig. 14b (30°C, 41% acetonitrile in water). Resolution is somewhat better than for the corresponding gradient separation ($R_s = 1.10$); this is generally true for isocratic vs. gradient elution, because of ''anomalous band broadening'' which occurs in gradient elution [11].

6. Conclusions

The present study has extended previous work [3] in further confirming that simultaneous changes in temperature and gradient time represent a promising procedure for optimizing band spacing and resolution in reversed-phase HPLC. Four initial experiments, where only temperature and gradient time are varied, allow the prediction and optimization of separation as a function of these two variables. This method-development approach is facilitated by the use of commercially available computer simulation soft-

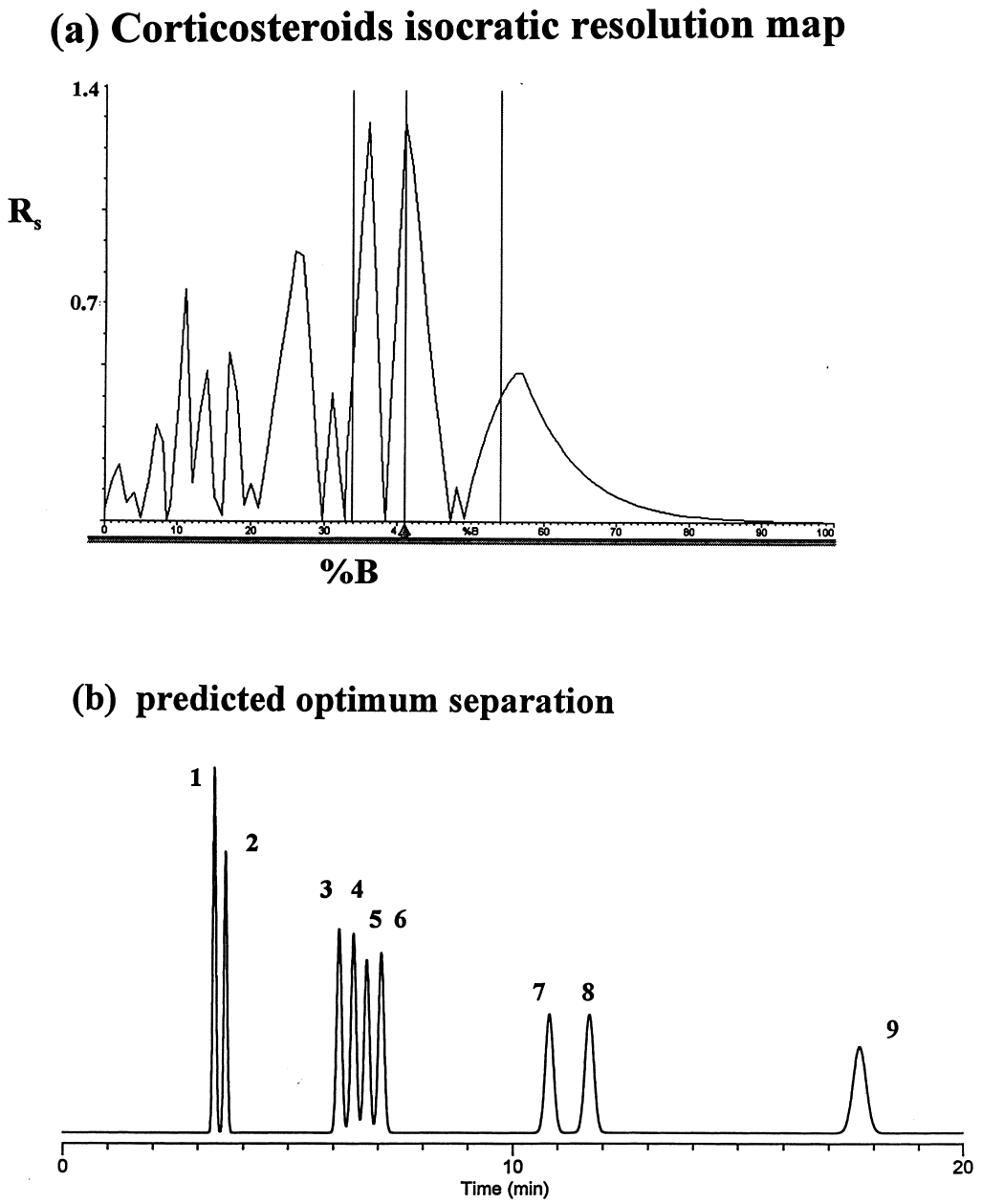


Fig. 14. Optimized isocratic separation of nine corticosteroids (sample 6 of Table 2). Conditions as in Fig. 7 except where noted otherwise. (a) Resolution map as a function of % B (% ACN) at 30°C; (b) predicted chromatogram for 41% B at 30°C.

ware as illustrated here. The separation of 10 samples in this way is described, and these results were augmented with previously reported results for four other samples. Of these 14 samples, nine could be separated with $R_s > 1.0$, and only two samples could not be separated with $R_s \geq 0.6$. This success rate is considered impressive, in view of the challenging nature of these 14 samples; five samples were

anticipated to be difficult to separate because of the chemical similarity of some or all of the components, and five samples contained ≥15 components. When the initial four experiments plus subsequent computer simulation fail to achieve a satisfactory result, further experiments as suggested in the following paper can be used to improve the separation. This sequential approach to method development minimizes the amount of work required for less demanding samples (i.e., only four method-development runs), provides a good start for the separation of more challenging samples, and avoids a number of potential problems in the final separation procedure. A similar approach (optimization of T and % B) for developing isocratic separations is also possible, based on the same four initial gradient runs.

7. Glossary of terms

The following list is for the present (Part I) and following (Part II [17]) papers. Defining equations are given for some terms (equation numbers include I- or II-, referring to Part I or II), and units are specified for those terms which are not dimensionless.

A, B	Solvent components of the mobile phase; A is usually water or buffer, and B is an organic solvent; also, constants in Eq. I-3 for a given solute where only temperature varies
ACN	Acetonitrile
b	Gradient steepness parameter (Eq. I-2)
d_p	Column-packing particle diameter (μm)
F	Mobile phase flow-rate (ml/min)
k	Retention factor
k^*	Effective retention factor in gradient elution (Eq. I-5)
k_w	Extrapolated value of k for water as mobile phase
L	Column length (cm)
LSD	Lysergic acid diethylamide
N	Column plate number
R_s	Resolution of two adjacent bands; R_s values for a sample containing more than two bands are for the most-overlapped band-pair
S	Solute parameter (Eq. I-1)
T	Temperature (°C)
THF	Tetrahydrofuran
t_G	Gradient time (min)
t_{G1}, t_{G2}	Values of t_G for two different input runs
t_0	Column dead-time (min)
V_D	Equipment dwell volume (ml)
V_m	Column dead-volume (ml)
α	Separation factor
$\Delta\varphi$	Change in φ during the gradient
φ	Volume-fraction of B solvent in mobile phase A/B; equal to 0.01% B
$\varphi_{i/j}$, $\varphi_{p/q}$	Values of φ at the column outlet at the time band-pair i/j or p/q leaves the column (Eq. II-1)
2D	Two-dimensional; referring here to temperature as the first dimension, and gradient time as the second dimension

Acknowledgements

The present study was supported in part by a Small Business Innovation Research (SBIR) grant from the National Institutes of Health (US Department of Health and Human Services).

References

[1] P.L. Zhu, J.W. Dolan, L.R. Snyder, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 756 (1996) 51.
[2] P.L. Zhu, J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, J.-T. Lin, L.C. Sander, L. Van Heukelem, J. Chromatogr. A 756 (1996) 63.
[3] L.R. Snyder, J.W. Dolan, I. Molnar, N.M. Djordjevic, LC·GC 15 (1997) 136.
[4] L.R. Snyder, J. Chromatogr. B 689 (1997) 105.
[5] P.L. Zhu, L.R. Snyder, J.W. Dolan, N.M. Djordjevic, D.W. Hill, L.C. Sander, T.J. Waeghe, J. Chromatogr. A 756 (1996) 21.
[6] L.R. Snyder, Today's Chemist at Work 5 (1996) 29.
[7] L.R. Snyder and M.A. Stadalius, in Cs. Horváth (Editor), High-Performance Liquid Chromatography – Advances and Perspectives, Vol. 4, Academic Press, New York, 1986, p. 195.
[8] L.R. Snyder, J.W. Dolan, D.C. Lommen, J. Chromatogr. 485 (1989) 65.
[9] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 485 (1989) 91.

[10] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 183.
[11] L.R. Snyder, J.W. Dolan, Adv. Chromatogr. 38 (1997) 115.
[12] P.L. Zhu, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 756 (1996) 41.
[13] L.R. Snyder, J.L. Glajch and J.J. Kirkland, Practical HPLC Method Development, Wiley-Interscience, New York, 2nd ed., 1997, Ch. 8.
[14] B.F.D. Ghrist, B.S. Cooperman, L.R. Snyder, J. Chromatogr. 459 (1989) 1.
[15] A.J. Sonderfan, M.P. Arlotto, D.R. Dutton, S.K. McMillen, A. Parkinson, Arch. Biochem. Biophys. 255 (1987) 27.
[16] Th. van der Hoeven, Anal. Biochem. 138 (1984) 57.
[17] J.W. Dolan, L.R. Snyder, D.L. Saunders, L. Van Heukelem, J. Chromatogr. A 803 (1998) 33.
[18] L.R. Snyder, M.A. Quarry, J. Liq. Chromatogr. 10 (1987) 1789.