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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CAROTENE AND VITAMIN A AND ITS GEOMETRIC ISOMERS IN FOODS

APPLICATIONS TO CHEESE ANALYSIS

BRUNO STANCHER* and FABIO ZONTA
Istituto di Merceologia, Università di Trieste, Via A. Valerio 6, 34127 Trieste (Italy)
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SUMMARY

A fractionation of retinol geometric isomers and of carotene was achieved, by straight-phase high-performance liquid chromatography, using a LiChrosorb Si 60 5-μm prepacked column and an isocratic mobile phase of methyl ethyl ketone–hexane (10:90). The wavelengths selected for detection of carotene and retinols were 450 nm and 340 nm respectively, and were changed during the chromatographic run. Two internal standards were used for quantitative analysis: 2-nitrofluorene for retinols and azobenzene for carotene. The proposed method, which has the advantage that all vitamin A active compounds can be evaluated simultaneously, was applied to the analysis of Italian cheese samples. The percentage recoveries of retinol and carotene were 75.7 and 79.6, respectively, and were not affected by cheese fat content, at least in the explored range.

INTRODUCTION

The biological activity of vitamin A should be referred to two large classes of compounds: retinoids and carotenoids[1].

Retinoids, such as retinyl aldehyde, retinol (usually referred to as vitamin A), retinyl esters, retinoic acid and its esters and related derivatives and metabolites (*e.g.*, 4-keto- or 4-hydroxyretinoic acid), as well as retinoid geometric isomers were previously determined by means of high-performance liquid chromatography (HPLC)[2–5]. HPLC was successfully been applied also in the separation of carotenoids, such as α- and β-carotene, cryptoxanthin and lycopene[6–9]. Some papers dealt with the separation of both carotenoids and retinoids, in an attempt to determine all vitamin A active compounds together[9–11].

In a previous note[12], we reported the HPLC separation of retinol isomers; this paper deals with a simultaneous, rapid separation of retinol isomers and carotene, suggesting a new HPLC method for assaying total vitamin A activity in food samples with a single analysis.

2-Nitrofluorene is used as an internal standard for determining retinol isomers,

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and azobenzene is proposed for carotene. The procedure has been developed as a tentative general method for food samples; an application to three different samples of Italian cheeses (selected with high, average and low fat contents) is presented. A study of the percentage recoveries of retinol and carotene was performed and total vitamin A activity in cheese samples was estimated.

EXPERIMENTAL

Apparatus

The liquid chromatographic apparatus was as previously described[12].

A prepacked HPLC column of LiChrosorb Si 60, 5 μm (250 × 4 mm) (E. Merck, Darmstadt, G.F.R.), equipped with a precolumn dry-packed with 40-μm silica pellicular packing (Supelco, Bellefonte, PA, U.S.A.) was employed.

Reagents and materials

All-*trans*-retinol (puriss.), 2-nitrofluorene (purum) and azobenzene (puriss.) were purchased from Fluka (Buchs, Switzerland). The β-carotene (Type III, crystalline, natural from carrots; 10–20% α-isomer, 80–90% β-isomer) was obtained from Sigma (St. Louis, MO, U.S.A.).

The hexane used for extractions was extrapure (96% GC, E. Merck). All other solvents (hexane, methyl ethyl ketone, ethanol) were HPLC grade (E. Merck).

L-(+)-Ascorbic acid (cryst. extra pure) (E. Merck), potassium hydroxide pellets (GR, E. Merck), anhydrous sodium sulphate (RPE; Carlo Erba, Milan, Italy) and sodium chloride (AnalaR; BDH, Poole, Great Britain) were also employed.

The working solutions of standards were prepared in hexane containing 10% (v/v) of methyl ethyl ketone (the same as the eluting phase). The concentration of the standard solution of retinol and carotene used for the calibration graphs was 3.00 mg all-*trans*-retinol plus 0.60 mg β-carotene per 100 ml. The internal standard solution was 2.00 mg 2-nitrofluorene plus 28.00 mg azobenzene per 100 ml.

Sample preparation

A 10-g amount of fresh cheese, ground in a food chopper or cut very finely, were digested in a 250-ml centrifuge tube with 25 ml of distilled water, 50 ml of ethanol and 25 ml of potassium hydroxide aqueous solution (100 g KOH in 100 g water). A 0.5-g amount of ascorbic acid was added as antioxidant as previously proposed[13,14]. The centrifuge tube was flushed with nitrogen, sealed with Parafilm® and occasionally shaken. After alkaline digestion overnight[15], the suspension was centrifuged at 2000 *g* for 15 min. The supernatant was poured into a 500-ml separatory funnel and extracted four times (shaking for 2 min) with 50-ml portions of hexane. The pooled extracts were washed twice with 50 ml of a saturated NaCl solution and once with 50 ml of distilled water. The washed organic layer was filtered through a Whatman No. 1 filter-paper (containing 30 g of anhydrous sodium sulphate) into a 250-ml flask, and the filter was rinsed twice with 10-ml portions of hexane.

A 2.50-ml volume of the internal standard solution (2-nitrofluorene plus azobenzene) was added to the flask. The solution was concentrated by means of a rotary vacuum evaporator (water-bath temperature $\leq 40^{\circ}C$) to a volume of about 2 ml. The concentrated solution was transferred quantitatively into a 5-ml volumetric flask,

which was then filled to the mark with a 10% (v/v) solution of methyl ethyl ketone in hexane. A 25-μl volume of the sample thus obtained was used for chromatographic injection.

Cheese samples were chosen from among those listed in the Italian Gazzetta Ufficiale (Italian legal gazette) and were selected according to their fat content. Thus Parmigiano Reggiano (fat content 32%), Montasio (40%) and Taleggio (48%) were analyzed, with a view to exploring samples containing the lowest, average and the highest fat contents, in order to establish its influence on the recovery of retinol and carotene.

Chromatographic procedure

The isocratic eluent mixture consisted of 10% (v/v) methyl ethyl ketone in hexane. With an operating flow of 1 ml/min, the pressure drop was 4.3–4.5 MPa at room temperature (25°C). The detection wavelength, λ, was 450 nm for carotene and azobenzene. After the elution of azobenzene, λ was quickly shifted to 340 nm in order to detect 2-nitrofluorene and retinol isomers. With an injection of 25 μl of cheese extract solution, the sensitivity setting was 0.1 a.u.f.s. The chart speed was adjusted to 10 mm/min or to 60 mm/min as required to compute peak areas.

Data processing

Statistical analysis of data was performed with an Olivetti P6040 desk calculator. The linearity of the calibration graphs was tested by the analysis of the variance (ANOVA) for the regression, using R^2 and F ratios as criteria of adequacy[16]. R^2 is the ratio between the sum of squares attributable to regression and the total sum of squares, and F is the ratio between the variance attributable to regression and the variance attributable to deviation from regression.

RESULTS AND DISCUSSION

Peak identification

Fig. 1 shows the chromatogram obtained with an injection of a test solution.

Peak identification of retinol, its isomers and 2-nitrofluorene has been accomplished according to the procedure described previously[12]. Owing to the fact that UV-cut-off of methyl ethyl ketone occurs at 330 nm, with this eluent it is obviously not possible to record UV spectra of eluting peaks at lower wavelengths. The identification of retinol isomer peaks was therefore based on the cited reference as well as on their relative retention times.

Carotene and azobenzene were identified by means of the stop flow method, recording their absorption spectra (see Fig. 2).

Internal standards

The use of 2-nitrofluorene as the internal standard for quantitation of retinol was discussed previously[12].

Azobenzene is proposed as internal standard for quantitation of carotene, since both these compounds show an absorbance maximum in the region of 450 nm and their chromatographic peaks are well resolved. The internal standard solution must be freshly prepared. On standing, azobenzene isomerizes into the *cis*-form which is detectable in chromatograms of aged internal standard solutions.

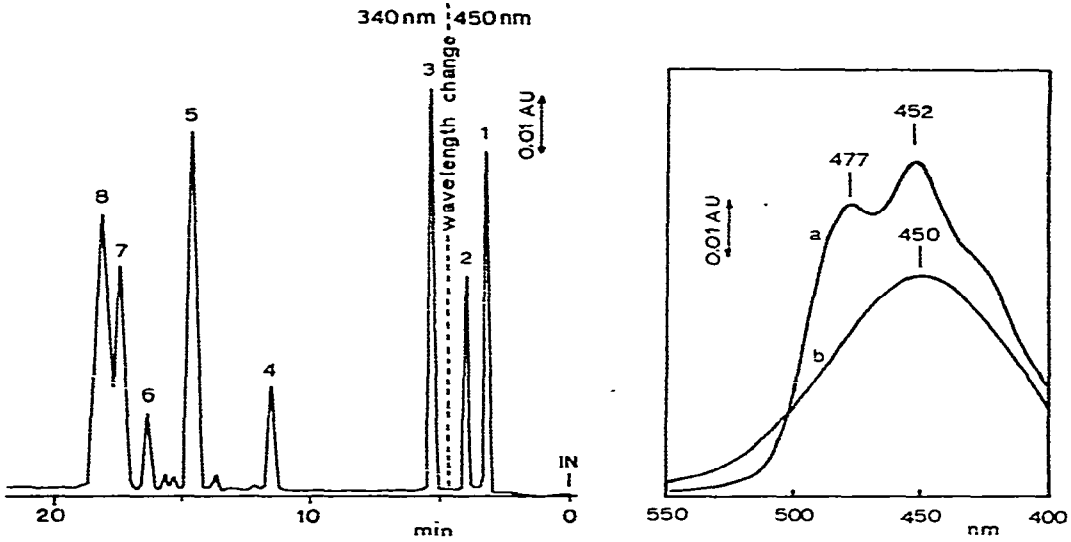

Fig. 1. Chromatogram of a test mixture of retinol geometric isomers, carotene and internal standards. Column: 5-μm Si 60 (250 × 4 mm) with precolumn, 40-μm pellicular silica (50 × 4 mm). Mobile phase: isocratic mixture of methyl ethyl ketone–hexane (10:90). Temperature: 25°C. Flow-rate: 1 ml/min. Pressure drop: 4.5 MPa. Detection wavelengths: 450 and 340 nm. Recorder sensitivity: 0.1 a.u.f.s. Chart speed: 10 mm/min. Peaks: 1 = carotene; 2 = azobenzene; 3 = 2-nitrofluorene; 4 = 11,13-di-*cis*-retinol; 5 = 13-*cis*-retinol; 6 = 9,13-di-*cis*-retinol; 7 = 9-*cis*-retinol; 8 = all-*trans*-retinol.

Fig. 2. Absorption spectra of β-carotene (a) and azobenzene (b). Spectra were recorded in a 8-μl spectrophotometer cell with the stop-flow method. Solvent system: methyl ethyl ketone–hexane (10:90).

Calibration graphs

Solutions used for construction of the calibration graphs were obtained as follows: aliquots of retinol plus carotene standard solution were added to 10-ml volumetric flasks containing 5.00 ml of internal standard solution; the flasks were then filled to the mark with 10% (v/v) methyl ethyl ketone in hexane. After chromatography of these solutions, calibration graphs were obtained by plotting the ratios A_R/A_{S1} and A_C/A_{S2} *versus* the concentrations of retinol and carotene respectively (A_R = retinol peak area; A_C = carotene peak area; A_{S1} = 2-nitrofluorene peak area; A_{S2} = azobenzene peak area).

The calibration graphs are shown in Fig. 3. They are linear over the explored range of concentration. Table I shows the values of the parameters a and b of the equation $y = a + bx$, where y is the ratio (analyte peak area/internal standard peak area) and x is the concentration, expressed in mg per 100 ml. The values of the standard deviation of the slope, s_b, and of the intercept, s_a, as well as F- and R^2-test values are also reported.

Cheese analysis

Analysis of cheese was performed by the standard-addition technique. Identical cheese samples were added of known quantities of retinol and carotene before starting the alkaline digestion. Analyses were carried out in quintuplicate, with four samples added with four different aliquots of analytes and one with no addition. Internal stan-

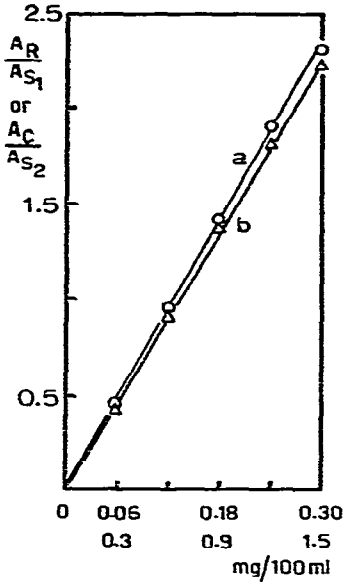

Fig. 3. Calibration graphs of carotene (a) and all-*trans*-retinol (b). Graph a shows A_C/A_{S2} *versus* concentration of carotene (upper scale on x axis); A_C = carotene peak area, A_{S2} = azobenzene peak area. Graph b shows A_R/A_{S1} *versus* concentration of retinol (lower scale on x axis); A_R = all-*trans*-retinol peak area, A_{S1} = 2-nitrofluorene peak area.

TABLE I

VALUES OF a, b, s_a, s_b, OF F AND R^2 TESTS AND OF PERCENTAGE RECOVERIES ($Q \pm s_Q$)

The values refer to the calibration graphs of retinol and carotene and to the standard-addition technique graphs of the analytes to three different cheese samples. As $y = a + bx$ is the general equation of the graphs, s_a and s_b represent the standard deviation of the intercept and of the slope respectively. s_Q is the standard deviation of the percentage recovery of analyte in the sample considered. Recovery values are obtained dividing b values of samples by the corresponding b value of the calibration graph. Cheese samples: 1 = Parmigiano Reggiano; 2 = Montasio; 3 = Taleggio.

	a	s_a	b	s_b	F	R^2	$Q \pm s_Q$
Retinol							
Calibration	0.001	0.022	1.503	0.022	4658	0.9993	
Cheese sample 1	0.666	0.024	1.135	0.025	2079	0.9985	75.5 ± 2.0
2	0.532	0.021	1.142	0.022	2756	0.9989	76.0 ± 1.8
3	0.566	0.024	1.138	0.024	2180	0.9986	75.7 ± 1.8
Carotene							
Calibration	0.012	0.016	7.766	0.080	2142	0.9986	
Cheese sample 1	2.230	0.028	6.153	0.143	1850	0.9984	79.2 ± 1.1
2	2.769	0.028	6.137	0.143	1838	0.9984	79.0 ± 1.0
3	1.864	0.038	6.270	0.156	1608	0.9981	80.7 ± 1.9

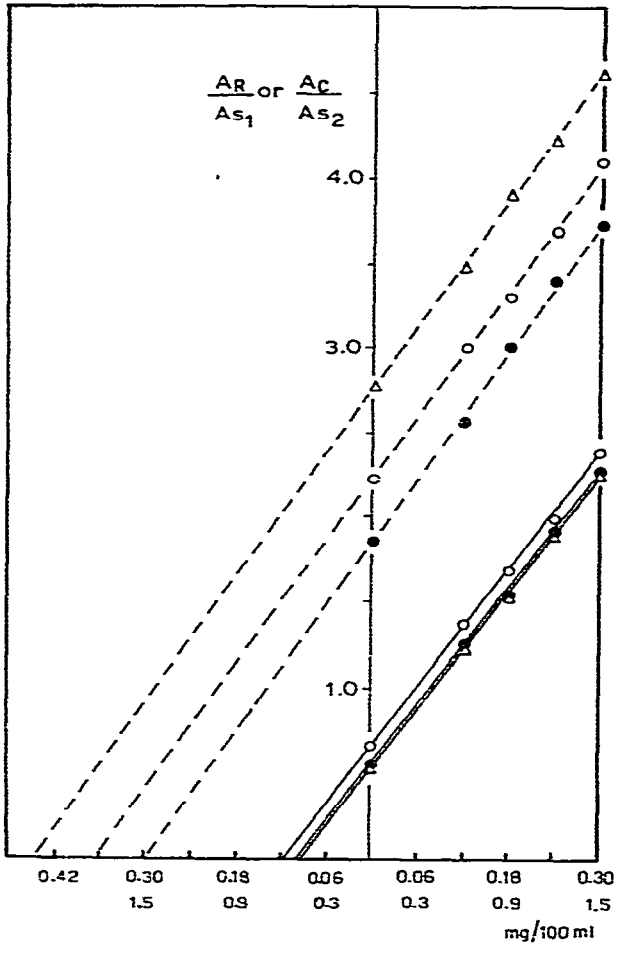

Fig. 4. Graphs obtained by means of the standard-addition technique of analytes to cheeses. x and y axes as in Fig. 3. Dotted lines refer to carotene, continuous lines to retinol. Cheeses: ○, Parmigiano Reggiano; ●, Taleggio; △, Montasio.

dards were also added (see *Sample preparation*) and at the end of the procedure the ratios A_R/A_{S1} and A_C/A_{S2} were plotted *versus* the known concentrations of added analytes. These graphs are shown in Fig. 4 and their parameters are listed in Table I. The quantities of retinol and carotene in a sample with no analytes added are then given by the intercepts of the graphs with the x axis.

Since the graphs obtained using the standard-addition technique were linear in the explored range of concentrations, the recovery of the analytes is unaffected by their concentrations. Furthermore, percentage recoveries of the analytes can be obtained by dividing the slope of these graphs by the slopes of the calibration graphs. Percentage recoveries were found to be unaffected by the fat content of cheese in the explored range (from 32 to 48%) since the slopes of the standard-addition graphs of different cheese samples were equal within the experimental error (see Table I). It follows that, in routine analysis of samples, it is possible to perform tests simply as

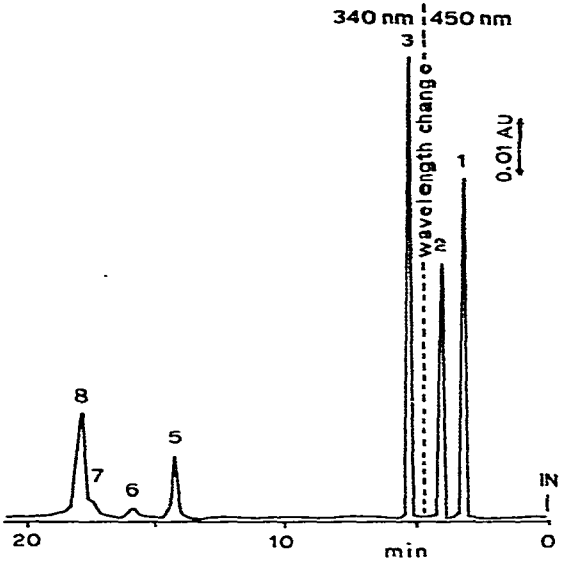

Fig. 5. Chromatogram of a Taleggio cheese extract. Chromatographic conditions and peak identification as in Fig. 1. 25 μl injected.

stated in *Sample preparation*. The quantities of analytes are obtained by comparing the values of A_R/A_{S1} and A_C/A_{S2} with those of the calibration graphs, and multiplying the obtained values by the known percentages recoveries.

Fig. 5 shows, as an example, the chromatogram obtained with the extract from a sample of Taleggio cheese. Peak 5 demonstrates the presence of 13-*cis*-retinol. Other retinol isomers (peak 6, 7) present in small amounts were not taken into account considering their low biological activity.

Retinols

Owing to the alkaline digestion of the sample, only retinols are detected because esters are completely hydrolyzed into alcohols. The completeness of this hydrolysis is indicated (as previously suggested[14]) by the lack of any ester peak in the chromatograms.

Since retinol geometric isomers have been found to have different biological activities[1], and the 13-*cis*-isomer has already been reported as naturally occurring in foods[14], it is advisable to quantify retinol separately from its isomers. The absorptivity ratio (1.08) of all-*trans*- to 13-*cis*-retinol at 328 nm has been used to calculate the 13-*cis*-retinol content[14]. Referring to available literature data[17,18], it can be seen that at 340 nm the absorptivity ratio of all-*trans*- to 13-*cis*-retinol is very close to 1.0. Therefore there is no need for any correction factor in order to quantitate these two isomers at this wavelength.

Carotenes

Owing to the great polarity difference between carotenes and retinols, the difficulty in resolving carotene isomers together with retinol isomers is not unexpected. Under the adopted chromatographic conditions, carotene is eluted very early,

TABLE II

CHEESE CONTENTS OF ALL-*trans*-RETINOL, 13-*cis*-RETINOL AND CAROTENE

Values for 13-*cis*-retinol and carotene equivalent to the biological activity of all-*trans*-retinol, as well as the resultant total vitamin A activity, are reported (in Italics). All values are expressed in μg per 100 g of fresh cheese.

Cheese	*Sample 1 (Parmigiano Reggiano)*	*Sample 2 (Montasio)*	*Sample 3 (Taleggio)*
All-*trans*-retinol	*293*	*233*	*249*
13-*cis*-Retinol	53	130	117
13-*cis*-Retinol × 0.75 (all-*trans*-retinol equivalents)	*39.7*	*97.5*	*87.7*
Carotene	181	225	149
Carotene × 1.6 (all-*trans*-retinol equivalents)	*30.1*	*37.5*	*24.8*
Total vitamin A activity	*363*	*368*	*361*

and no separation of α- and β-carotene can be achieved. Therefore only total carotene is determined with the proposed method.

Evaluation of total vitamin A active compounds

13-*cis*-Retinol is only 75% as active as all-*trans*-retinol[1], and it is necessary to multiply the weight of the former by 0.75 to obtain the equivalent weight of the latter. Vitamin A activity is then given by:

$$\mu g \text{ of retinol equivalents} = \mu g \text{ of all-}trans\text{-retinol} + 0.75 \cdot \mu g \text{ of 13-}cis\text{-retinol}$$

Considering only the more common and wide spread carotenoids, those which exhibit a vitamin A activity in mammals can be narrowed down to three species: α-carotene, β-carotene and cryptoxanthin[6]. To evaluate their vitamin A activity, the following formula was proposed[7]:

$$\mu g \text{ of retinol equivalents} = (\mu g\ \beta\text{-carotene})/6 + (\mu g\ \alpha\text{-carotene})/12 + (\mu g\ \beta\text{-cryptoxanthin})/12.$$

Parrish[19], referring specifically to margarine and butter, took into account only total carotene, and the above formula thus simplifies to: μg of retinol equivalents = (μg carotene)/6.

Total vitamin A activity, of retinols and carotene is then given by:

$$\mu g \text{ of retinol equivalents} = \mu g \text{ of all-}trans\text{-retinol} + 0.75 \cdot \mu g \text{ of 13-}cis\text{-retinol} + (\mu g \text{ of carotene})/6.$$

Table II reports the values for all-*trans*-retinol, 13-*cis*-retinol and carotene, and the resulting total vitamin A activity found in the considered cheese samples.

REFERENCES

1 W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. II, Academic Press New York, 2nd ed., 1967.
2 C. D. B. Bridges, S. L. Fong and R. A. Alvarez, *Vision Res.*, 20 (1980) 355.
3 B. A. Halley and E. C. Nelson, *J. Chromatogr.*, 175 (1979) 113.
4 J. E. Paanakker and G. W. T. Groenendijk, *J. Chromatogr.*, 168 (1979) 125.
5 A. M. McCormick, J. L. Napoli and H. F. DeLuca, *Methods Enzymol.*, 67 (1980) 220.
6 S. K. Reeder and G. L. Park, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 595.
7 I. Stewart, *J. Ass. Offic. Anal. Chem.*, 60 (1977) 132.
8 A. Fiksdahl, J. T. Mortensen and S. Liaaen-Jensen, *J. Chromatogr.*, 157 (1978) 111.
9 T. Van de Weerdhof, M. L. Wiersum and H. Reissenweber, *J. Chromatogr.*, 83 (1973) 455.
10 W. O. Landen, Jr. and R. R. Eitenmiller, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 283.
11 W. O. Landen, Jr., *J. Chromatogr.*, 211 (1981) 155.
12 B. Stancher and F. Zonta, *J. Chromatogr.*, 234 (1982) 244.
13 D. B. Parrish, *CRC Crit. Rev. Food Sci. Nutr.*, 9 (1977) 375.
14 D. C. Egberg, J. C. Heroff and R. H. Potter, *J. Agr. Food Chem.* 25 (1977) 1127.
15 J. N. Thompson, W. B. Maxwell and M. L'Abbe', *J. Ass. Offic. Anal. Chem.*, 60 (1977) 998.
16 N. R. Draper and H. Smith, *Applied Regression Analysis*, Wiley, New York, 1966, 7.
17 R. Hubbard, *J. Amer. Chem. Soc.*, 78 (1956) 4662.
18 C. D. Robeson, J. D. Cawley, L. Weisler, M. H. Stern, G. C. Eddinger and A. J. Chechak, *J. Amer. Chem. Soc.*, 77 (1955) 4111.
19 D. B. Parrish, *J. Ass. Offic. Anal. Chem.*, 63 (1980) 468.