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Analytical Methods

Photoacoustic spectroscopy and optothermal window as analytical tools to quantitate carotenes in margarines

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Abstract

Photoacoustic spectroscopy (PAS) and optothermal window (OW) with a cw Ar ion laser used as a radiation source at 476.5, 488 and 496 nm were applied to quantify total carotenes (TC) in margarines. Both techniques, being rapid and extremely simple, allow for direct measurement without any pretreatment of the sample. The PAS has proven precise and sensitive enough to allow quantitation of TC in margarines containing 1–9 mg TC/kg, in applications such as the quality control of intermediate and final products. The sensitivity of OW is lower than that of PAS but the approach can still be used for quantitation of TC in margarine matrices at much higher concentrations.

Keywords: Total carotenes; Margarines; Photoacoustic spectroscopy; Optothermal window; Spectrophotometry; Quantitation

1. Introduction

Beta carotene, a carotenoid commonly known as provitamin A, has in the last decades become even more important as an antioxidant, namely, as a radical scavenger and a physical scavenger of singlet oxygen (Burton, 1989; Krinsky, 1989). It is believed that β-carotene plays an important role in the inhibition of initial stages of lipid peroxidation and possibly also possesses anticancer properties (Bauernfeind, 1981; Krinsky, 1989). The protective effect of carotenes added as colorants to various foods has been the part of exhaustive epidemiological, nutritional and food quality research (Goodwin, 1986). HPLC has been often employed as a powerful technique to quantify low levels and various forms of carotenoids in foods and in human plasma (Khachik, Beecher, & Whittaker, 1986; Khachik, Beecher, Goli, Lusby, & Smith, 1992).

Margarine compositions are coloured by the incorporation of a carotenoid pigment having a yellowish hue due to α-carotene, e.g., palm oil or saffron or carrot extract, or β-carotene, along with a natural material having a predominantly reddish hue, e.g., annatto or paprika, sufficient to produce a yellow colour. It was found by Van het Hof, Tijburg, de Boer, Wiseman, and Weststrate (1998) that the consumption of the antioxidant-fortified margarine significantly increased the levels of supplied antioxidants in plasma, with the largest increase found for α- and β-carotene.

According to Fox and Minchinton (1972) the most common colorant found in margarines is synthetic β-carotene. The same authors found 1–13 mg/kg of carotene in coloured margarine as opposed to 3.9 mg carotene/kg in margarine reported by Toyos Diaz (1968). Several methods have been used over the past decades to determine carotene colorants in margarines. Saponification and extraction were often followed by chromatographic separation and spectrophotometric/chromatographic determination (Fox & Minchinton, 1972; Maruyama, Ushigusa, Kanematsu,

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Niiya, & Imamura, 1977; Toyos Diaz, 1968; Usher, Favell, & Lavery, 1968). It should be kept in mind that saponification step not only prolongs the analysis time but in addition may also cause degradation of carotenoids. RP-HPLC method was developed for separation of vitamin A and β-carotene in oil and margarine (Landen & Eitenmiller, 1979) and for quantitation of these compounds in margarine after gel permeation chromatographic separation (Chase, Akoh, Eitenmiller, & Landen, 1995). The need for availability of accurate qualitative and quantitative data on carotenoids has resulted in a development of rugged analytical techniques capable of separating, identifying and quantifying these compounds. Recently, we have proposed a direct spectrophotometric method for assaying total carotenes in margarines after a simple extraction in hexane (thereby obviating the saponification step) and discussed in-extenso its analytical performances (Luterotti, Bicanic, & Pozgaj, 2006).

On the other hand the photoacoustic spectroscopy (PAS) and optothermal window (OW) concept with laser used as the excitation source have already proven effective analytical methods for quantification of lutein in biological matrix (Bicanic, Luterotti, Becucci, Fogliano, & Versloot, 2005) and of lycopene in tomato-based products (Bicanic et al., 2003, 2004, 2005). The research study described in this paper explores the capability of PAS and OW for simple and direct (without a need for any sample pretreatment step) quantification of total carotenes (TC) in margarine matrices.

The principles of PAS and OW rely on the transfer of the periodically modulated radiation to a heat in the absorbing condensed phase sample. The heating and cooling that takes place in response to the incident radiation is in PAS converted into a pressure wave which, in a constant-volume chamber, is detected by a microphone at the modulation frequency. On the other hand, in OW, the heat generated on account of absorption in the sample causes the periodic expansion/contraction in a radial direction of a thin sapphire plate (in a good thermal contact with the sample) which is sensed at the modulation frequency by the piezoelectric device attached to the rear side of disk. For a given incident power of the selective excitation source, the intensities of PA and OW signals from microphone and piezoelectric sensor are proportional to the sample concentration. Hence PAS and OW can be conveniently applied in studies on completely opaque samples making them unique in the comparison with conventional spectroscopy where specimen's transparency is a necessity.

2. Materials and methods

2.1. Samples and standard

Overall, 27 margarines manufactured and purchased (some were donated by "Zvijezda", Zagreb, Croatia) in different European countries were investigated. Eighteen among them contained an aqueous phase (water-containing margarines, abbreviated WCM, and water-containing blanks, abbreviated WCB), the remaining ones contained only the fat phase (fat margarines, abbreviated FM, and fat blanks, abbreviated FB). Overall, eight blanks (no carotene colorant added) were used: three with water added (see above WCB) and five without water added (see above FB).

All the above groups of margarines were classified and screened according to their total fat content: low fat margarines (abbreviated LF, 34–40% total fat) and high fat margarines (abbreviated HF, 60–94% total fat). The fat content and composition of the margarines were provided by the manufacturer. Codes and composition of the margarines are given in Table 1.

Unlike OW that was used only with WCM, PAS was applied in studies on all types of samples, namely, WCM, WCB, FM and FB. The samples were homogenized and kept at +4 °C; before actual measurement they were equilibrated at ambient temperature.

Betatene® 20% Soy (Cognis Australia Pty Ltd., Cheltenham, Vic., Australia), the gift of Dr. Christina Gaertner from Cognis GmbH, Düsseldorf, Germany, was used for standard addition of carotenes. It was stored at −18 °C. For standard addition experiments an accurately weighed mass of Betatene® was mixed into sample **4** and exhaustively triturated.

Table 1
Margarines classified by type and composition

Margarine code	Margarine type		Total fats (%)	Saturated/unsaturated fatty acids ratio
	Carotenes added	Aqueous phase added		
1 (WCB_{HF})	−	+	80	1:2
2 (WCB_{LF})	−	+	40	1:3
3 (WCB_{HF})	−	+	60	1:2
4 (WCM_{HF})	+	+	94	1:2
5 (WCM_{HF})	+	+	80	1:2
6 (WCM_{HF})	+	+	80	1:2
7 (WCM_{HF})	+	+	80	1:2
8 (WCM_{HF})	+	+	80	1:2
9 (WCM_{HF})	+	+	63	1:2
10 (WCM_{LF})	+	+	40	1:3
11 (WCM_{LF})	+	+	40	1:2
12 (WCM_{LF})	+	+	38	1:3
13 (WCM_{LF})	+	+	38	1:3
14 (WCM_{LF})	+	+	34	1:3
15 (WCM_{LF})	+	+	40	1:3
16 (WCM_{HF})	+	+	60	1:2
17 (WCM_{HF})	+	+	60	1:2
18 (WCM_{LF})	+	+	40	1:3
19 (FB_{HF})	−	−	80	1:2
20 (FB_{HF})	−	−	63	1:2
21 (FB_{LF})	−	−	40	1:3
22 (FB_{LF})	−	−	40	1:3
23 (FB_{HF})	−	−	60	1:2
24 (FM_{HF})	+	−	80	1:2
25 (FM_{HF})	+	−	63	1:2
26 (FM_{LF})	+	−	40	1:3
27 (FM_{HF})	+	−	60	1:2

WCB – water-containing blank, FB – fat blank, WCM – water-containing margarine, FM – fat margarine, LF – low fat, HF – high fat.

2.2. Spectrophotometry (SP)

Total carotenes (TC) in margarines were assayed by the spectrophotometric method (Luterotti et al., 2006). Each sample was analyzed independently 6 to 18 times and mean ± SD values were presented. Results for the samples investigated in this study were collected over a period longer than a month.

2.3. Photoacoustic (PAS) measurements

Measurements were performed at the room temperature at three wavelengths: 496 (emitted power 10 or 15 mW), 488 (emitted power 15 or 20 mW) and 476.5 nm (emitted power 10 mW) of a cw argon laser (Lexel 85, Freemont, California, USA). Laser power reported within the brackets refers to that incident on the sample in the PAS cell and was measured with portable power detector, model 407 A, from Spectra Physics (Irvine, California, USA). The modulation frequency (24 or 25 Hz) was achieved by means of the SR540 mechanical chopper (Stanford Research Corporation, Sunnyvale, California, USA). At this modulation frequency thermal diffusion length in margarine is estimated to about 175 μm. The non-resonant PAS cell was a home-made device equipped with the Suprasil window, "O" ring seal and the KE 4-211-2 Sennheiser microphone (Sennheiser, Wedemark, Germany). The sample holder, the stainless steel plate (50 mm in diameter) provided with a central semispherical cavity (diameter 8 mm), served to accommodate specimen of margarine. This cavity was loaded flush with the flat surface of the plate using a spatula. Laser beam was not focused; at the location of the test product the diameter of the laser spot was about 2 mm. Under these conditions the transfer of thermal energy in the lateral direction is negligible and therefore the flow of heat can be treated as being one-dimensional. The PAS signal from a microphone was detected at the modulation frequency with the EG&G 2101 two phase lock-in amplifier (Princeton Applied Research/EG&G, Oak Ridge, Tennessee, USA) using an integration time of 1 s; both the amplitude and the phase of the PAS signal were recorded. The sampling rate of the lock-in amplifier was 60 data in 45 s. The PAS signals were divided (normalized) to those obtained under the same experimental conditions from the black drawing ink (Rotring); these normalized signals were then used to calculate product of thermal diffusion length μ and absorption coefficient per unit length β of the product being measured (Bicanic et al., 2003). Data was collected over a 15-months period. Results are presented as a mean ± SD of 2–9 independent analyses for each set of experimental parameters.

2.4. Optothermal window (OW) measurements

Measurements were performed at ambient temperature. The experimental set-up consisted of a cw Ar ion laser (Lexel 85), EG&G 2101 two phase lock-in amplifier (Princeton Applied Research), the SR540 chopper (Stanford Research Systems) and the home-made OW device. This latter incorporates a thin sapphire window with the piezodetector glued to its rear face. The measurements were carried at 488 nm (using 30 mW) laser line, frequency of 25 Hz and the lock-in amplifier integration time of 1 s. Thus obtained gross (raw) OW signals were corrected to compensate for the background absorption; this was conveniently accomplished by measuring OW signal from the sapphire window when loaded only with water. This background signal was then algebraically subtracted from the gross (raw) OW signals in order to get the net OW signals for each sample. These latter were finally normalized by dividing them with OW signals obtained from a black ink (very strong absorber that serves as a reference) and eventually converted into the product of thermal diffusion length μ and absorption coefficient per unit length β as described before (Bicanic et al., 2003). Results collected over a period of few days are presented as a mean ± SD of 3–9 independent analyses.

3. Results and discussion

The absorption spectrum of β-carotene shows peaks at approximately 450 and 480 nm. Consequently, 496, 488 and 476.5 nm emission lines of Ar ion laser appear suitable for excitation of this analyte in the PAS and OW measurements.

3.1. Correlations PA or OW versus SP

We have found that both, raw and normalized PAS signals exhibit identical correlation coefficients (R) versus concentration of total carotenes (TC); however the slopes and intercepts were different. Normalization improved accuracy by eliminating the systematic error without affecting the extent of correlation. However in the OW experiments the normalization markedly extended the linear concentration range.

Concentration of total carotenes in the test samples, determined spectrophotometrically according to Luterotti et al. (2006), ranged between 1.3 and 9.0 mg/kg. As to the PAS, the relationship found between product $\beta\mu$ and TC concentration was linear after grouping margarines according to the presence of water. As far as FMs are concerned the linearity extends up to 9 mg TC/kg. This upper concentration limit reduces to 8.5 mg/kg if: (i) the correlation plot includes all samples and (ii) for water-containing samples prior to their classification based on the total fat content (Fig. 1).

No correlation up to 9 mg TC/kg was found when OW was applied; this regardless of classifying margarines on the basis of their total fat content. However, good linear correlation was achieved (Fig. 2) for higher concentrations (up to 640 mg TC/kg) with R of 0.995 and 9.9×10^{-3} as residual sum of the squares (RSS). This makes OW applicable for calibration in margarine matrices only at TC levels much higher than those found in table margarines.

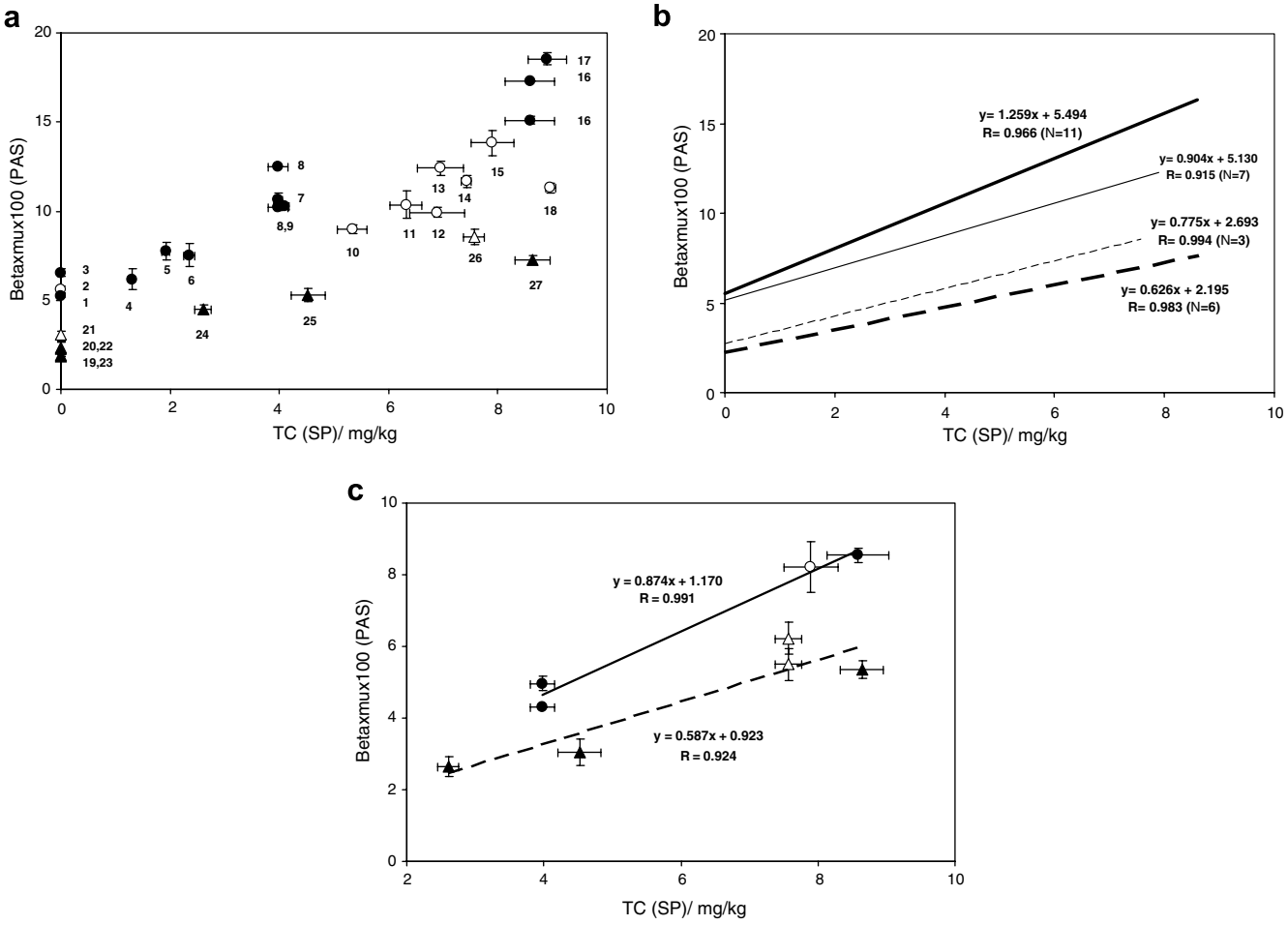

Fig. 1. Hundredfold dimensionless product of the absorption coefficient per unit length β and thermal diffusion length μ obtained from PAS experiments plotted versus the concentration of TC estimated spectrophotometrically (SP). Experimental conditions: laser wavelength 476.5 nm, laser power 10 mW, modulation frequency 24 Hz, integration time of lock-in amplifier 1 s. Each data point represents the mean ± SD of 2 to 9 independent PAS results and 6 to 18 independent SP results. (a) Individual margarines, all types, $N = 29$; (b) regression lines for each type of margarine (based upon points from a); (c) regression lines for blank-corrected PAS signals (see Table 2). Symbols: water-containing samples (all, ———), (high fat, ● or ———), (low fat, ○ or ———); fat samples (all, – – – –), (high fat, ▲ or ▬ ▬), (low fat, △ or ------).

As far as PAS is being concerned, the correlation improved substantially when margarines were classified according to the presence of water. The classification based on the total fat content not only further enhanced the correlation parameters (R, RSS) between $\beta\mu$ and TC concentration but also extended the linearity range. In general, correlation was better at lower laser power. The effect of wavelength selection on the extent of correlation was not observed. For example, at 496 nm coefficient of correlation ranged between 0.89 and 0.95, at 488 nm between 0.92 and 0.95, and at 476.5 nm between 0.81 and 0.99. In the latter case, R, for all samples taken together, water-containing samples and fat samples was 0.81, 0.90 and 0.97, respectively. Additional classification based on the total fat content improved R for water-containing samples even to 0.97 and for fat samples to 0.99 (see Table 2). Data for fat samples were less scattered than that from the water-containing ones. Overall, the total fat content affects the correlation less than the presence of water does. Correcting brutto PAS signals by subtracting PAS signals from the respective blank margarine did not yield substantial improvement of correlation coefficient (Table 2 and Fig. 1c). Correlation data is presented in Table 2.

3.2. Performance characteristics of PAS

The limit of detection achievable by PAS might be lowered to below 2 mg TC/kg. However, the working concentration range extending up to 9 mg TC/kg makes PAS suitable for estimating TC concentration in commercial margarines.

Precision of TC quantitation by PAS is evidenced upon within-a-run repeatability and intermediate precision data (between a couple of days) (Table 3).

Achieved precision of PAS data is comparable for all investigated wavelengths. Favourable precision of TC analysis is evident and ranges between 2% and 9%; the increase in total fat content lowered the precision. The repeatability attained with the above described PAS setup, upon single load measurements (60 readings in 45 s) was excellent with

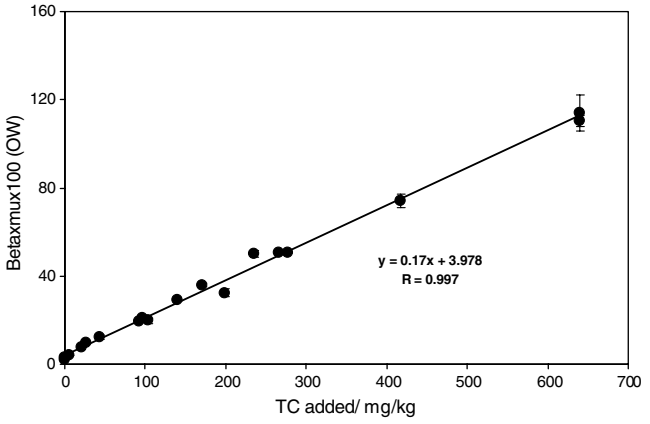

Fig. 2. Hundredfold dimensionless product of the absorption coefficient per unit length β and thermal diffusion length μ obtained from OW experiments plotted versus the concentration of TC added to sample **4** ($N = 18$). Standard additions (mg TC/kg): 5.9, 21.4, 27.1, 43.4, 92.8, 96.6, 103.3, 140.6, 171.4, 199.1, 234.8, 265.4, 277.5, 417.2, 640.4. Experimental conditions: laser wavelength 488 nm, laser power 30 mW, modulation frequency 25 Hz, integration time of lock-in amplifier 1 s. Each point represents the mean ± SD from 2 to 17 independent OW measurements.

Table 3
Precision data for PAS measurements[a]

Laser excitation wavelength λ (nm)		496 (10 mW and 15 mW)	488 (15 mW and 20 mW)	476.5 (10 mW)
Repeatability	WCM[b]	1.4–5.7	1.4–9.5	1.4–9.4
	FM[b]	–	–	3.3–7.1
	WCB[c]	1.6–2.7	2.5–3.0	1.8–3.4
	FB[d]	–	–	3.2–18.2
Intermediate precision	WCM[e]	2.6–7.0	–	6.1–9.0
	FM	–	–	–
	WCB[f]	6.6	–	–
	FB	–	–	–
Instrument repeatability	WCM[g]	–	–	0.3–0.7
	FM	–	–	–
	WCB[h]	–	–	0.7
	FB	–	–	–

WCB – water-containing blank, FB – fat blank, WCM – water-containing margarine, FM – fat margarine.
(–) – not enough data available for the calculation.
Number of independent analyses (multiple loadings): [b]$n = 3$–9, [c]$n = 3$–4, [d]$n = 4$–6, [e]$n = 5$–7, [f]$n = 7$.
Number of repeated recordings of the instrument (single loading of the sample): [g]$n = 5$–6, [h]$n = 5$.
[a] Relative standard deviation (%), RSD.

RSD not exceeding 0.7%. In the case of OW measurements high imprecision (up to 44%) might be expected at TC concentration below 2 mg/kg, in the concentration range 2–9 mg/kg the precision is better (1.4–11.9%) and improves further (0–7.3%) at still higher concentrations (20–640 mg/kg).

3.3. Influence of margarine nature on the PAS signal

The magnitude of the PAS signal strongly depends on the composition of the margarine, in particular on the presence of water and the total fat content in the sample. Both parameters influence the thermal properties of margarine and hence the PAS signal as well. In LF samples, i.e., those with ⩽40% total fats, ratio of saturated-to-unsaturated fatty acids (except for sample **10**) was 1:3, whereas in HF samples, namely those containing ⩾60% total fats, the ratio of saturated-to-unsaturated fatty acids was 1:2.

Even the blanks, both water-containing and the fat ones, produced high PAS signals. Water-containing samples including blanks showed significantly larger signals than the fat samples. For example, the signals from WCBs were several times higher than those from FBs; e.g., the value of product 100 $\beta\mu$ ranged from 2 to 3 for FBs and from 5 to 10 in case of WCBs. The parasitic PAS signal at zero TC concentration is clearly observed in the correlation plots (see Fig. 1a and b). The slopes of correlation plots increased from HF fat samples, through LF fat and LF water-containing samples to HF water-containing samples (Fig. 1b). Namely, lower fat content is accompanied by the smaller slope in water-containing margarines, in case of fat

Table 2
Correlations of PAS versus SP data

Laser excitation wavelength λ (nm)		496 (10 mW and 15 mW)[a]	488 (15 mW and 20 mW)[a]	476.5 (10 mW)[a]	
				Uncorrected	Blank-corrected
Water-containing + fat samples	All	–	$0.930/2.9 \times 10^{-3}$ (19)	$0.813/1.5 \times 10^{-2}$ (27)	$0.806/1.2 \times 10^{-3}$ (9)
	LF	–	–	$0.915/3.5 \times 10^{-3}$ (10)	$0.966/2.6 \times 10^{-5}$ (3)
	HF	–	–	$0.771/1.3 \times 10^{-2}$ (17)	$0.798/8.1 \times 10^{-4}$ (6)
Water-containing samples	All	$0.899/2.8 \times 10^{-3}$ (15)	$0.930/2.1 \times 10^{-3}$ (18)	$0.896/5.3 \times 10^{-3}$ (19)	$0.991/2.6 \times 10^{-5}$ (4)
	LF	$0.949/6.5 \times 10^{-4}$ (6)	$0.921/1.2 \times 10^{-3}$ (9)	$0.915/7.0 \times 10^{-4}$ (7)	–
	HF	$0.887/1.6 \times 10^{-3}$ (9)	$0.948/7.4 \times 10^{-4}$ (9)	$0.966/1.0 \times 10^{-3}$ (11)	$0.989/2.2 \times 10^{-5}$ (3)
Fat samples	All	–	–	$0.967/3.1 \times 10^{-4}$ (9)	$0.924/1.5 \times 10^{-4}$ (5)
	LF	–	–	$0.994/2.6 \times 10^{-5}$ (3)	–
	HF	–	–	$0.983/8.2 \times 10^{-5}$ (6)	$0.984/1.4 \times 10^{-5}$ (3)

LF – low fat, HF – high fat.
(–) – not enough data for the calculation.
[a] Correlation data are given through correlation coefficient (R) and residual sum of the squares (RSS) values, R/RSS. Number of points obtained by multiple independent analyses are given in parentheses.

margarines the situation is just opposite. For both, water-containing and fat samples, the intercept for LF and HF samples was practically the same (Fig. 1b).

3.4. Applicability of PAS and OW

It was shown that PAS can be used for fast and simple quantitation of total carotenes in margarines. In practice, PAS can be applied to control the manufacturing process of margarine itself (fat margarines) as well to inspect final products (water-containing margarines). Worth mentioning is the fact that margarines with both, low as well as high total fat content (from 34% to as high as 94%), could be measured. For the purpose of quantitation the calibration line for respective type of margarine should be constructed up to 9 mg TC/kg, a range wide enough to cover the typical concentrations of carotenes in table margarines. The OW approach is not sensitive enough to allow detection of TC at a level typically found in household margarines but it can still be successfully used to determine TC in highly carotene-enriched margarine matrices.

4. Conclusion

In conclusion, PAS is proposed as a new analytical tools for a simple and rapid, routine control of TC concentration in commercial margarines. With the present state of art in development of strong radiation sources and required electronic equipment, it is possible to manufacture a versatile PAS-based instrument at only moderate investment cost. For proper interpretation of measured data classifying the margarines according to (i) the presence of water, (ii) total fat/water content, is a necessity.

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