

Determination of chlorophyll in plant samples by liquid chromatography using zinc–phthalocyanine as an internal standard

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Abstract

Chlorophyll analysis at high precision and accuracy is limited by the lack of suitable, commercially available internal standards for HPLC analysis. Here, the commercially available dye zinc–phthalocyanine is presented as a new internal standard to quantify chlorophylls in vegetable foods and to detect chlorophyll degradation products. The technique was applied to chlorophyll analysis of a selection of vegetable foods. Pigments were extracted with *N,N*-dimethylformamide from the vegetables and purified by solid phase extraction. Chlorophyll *a*, *a'*, *b*, *b'*, corresponding pheophytins, and zinc–phthalocyanine were separated by HPLC using a C₁₈ reverse-phase column and fluorescence detection. © 2003 Elsevier B.V. All rights reserved.

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1. Introduction

Quantitative analysis of chlorophylls and their degradation products is performed in a number of scientific disciplines, e.g. in oceanography for monitoring phytoplankton growth in seawater [1]. In food science, chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) and their copper complexes are of interest as food dyes (E 140, E 141). In addition, chlorophylls and their degradation products are used as markers of food processing as chlorophylls are sensitive to light, pH, temperature, oxygen and enzymatic degradation [2,3].

Spectrophotometry [4,5] and fluorometry [6,7] are the techniques most commonly used for chlorophyll quantification. Fluorescence methods are more sensitive and selective than spectrophotometric techniques for chlorophyll but of limited accuracy when applied to complex sample matrices because of unpredictable quenching effects [8]. Furthermore, chlorophylls have to be present in non-aqueous solutions as fluorescence activity decreases with increasing amounts of water [9]. For both spectroscopic techniques, chlorophylls are usually separated from the sample matrix to

remove spectral interferences [10,11]. HPLC has established itself as the most powerful tool for this purpose [12,13].

Pigment quantification is usually done by external calibration or via compound specific absorption coefficients. Using these techniques, the accuracy and precision of the analysis can be limited by the often considerable analyte losses that may occur during sample preparation. This limitation can be overcome by using an internal standard. Provided that there is no discrimination between analyte and internal standard during sample preparation, analyte losses do not affect the accuracy of the analysis. Despite these advantages, internal standards are rarely used for chlorophyll analysis because of the lack of chemically similar compounds that are stable during storage, pure, and commercially available. Bessiere and Montiel [14] used fluoranthene as an internal standard to determine chl *a* and chl *b* in phytoplankton by HPLC and fluorescence detection. However, fluoranthene differs chemically from the chlorophyll pigments which increases the risk of differences in pigment degradation during extraction and purification. The same is true for sudan II [15] and β -apo-8'-carotenal [16]. Some potentially useful internal fluorescence standards have been discussed by Mantoura and Repeta [17] but standards were either relatively expensive such as mesoporphyrin IX dimethylester [18], co-eluted with the pigments (etioporphyrin or deuteroporphyrin IX dimethylester) or were not commercially available (zinc-pyropheophorbide *a*). As a consequence,

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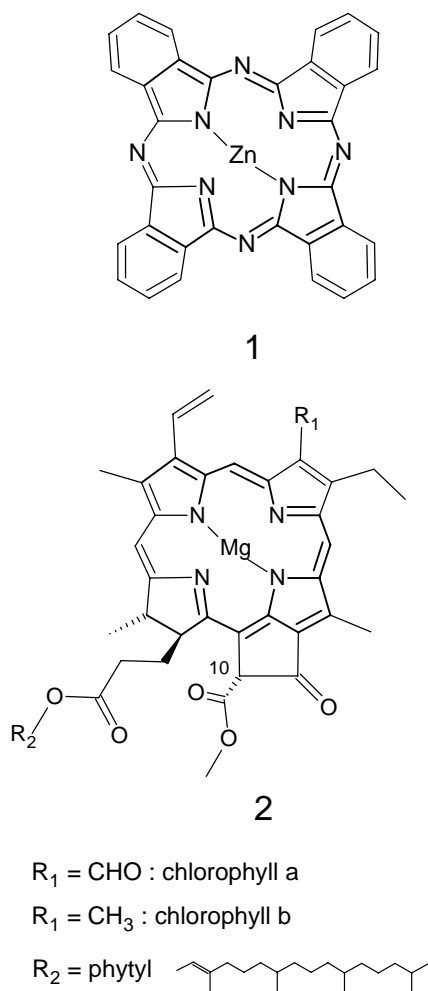


Fig. 1. Structure of zinc-phthalocyanine (1), chlorophyll *a* and chlorophyll *b* (2).

laboratories are usually obliged to synthesize their own internal standards or use external calibration methods.

Considering previous approaches, alternative compounds were evaluated for their usefulness as internal fluorescence standards. Here, we show that zinc-phthalocyanine, a commercially available dye used in food industry and cancer research, can be used as an internal HPLC standard for routine analysis of chl *a* and chl *b* in plant foods. Zinc-phthalocyanine (Fig. 1) shows structural and spectroscopic similarities to the chlorophylls, is relatively cheap, stable, and can be purchased at sufficient purity.

2. Experimental section

2.1. Standards and chemicals

Chl *a* and chl *b* (Fluka, Buchs, Switzerland, certified dye content >95%), and zinc-phthalocyanine (Aldrich, Buchs, Switzerland, certified dye content >97%) were purchased in solid form. The purity of these pigments was

verified by HPLC. Reagents and solvents were of analytical grade unless otherwise specified. Chlorophyll standards were prepared by dissolving 5 mg chl *a* and 5 mg chl *b* in 50 g acetone (Merck), respectively, and 30 mg zinc-phthalocyanine in 100 g *N,N*-dimethylformamide (DMF) (Fluka). Standards were prepared gravimetrically by weighing reagents to ± 0.05 mg and dissolution in cooled solvents (5 °C). Pheophytin standards were obtained from the chl *a* and chl *b* standards by acidifying 20 ml aliquots with 0.5 ml 1 M HCl. Standard solutions were stored in the dark under argon at -25 °C. In addition to zinc-phthalocyanine, other compounds were tested for their usefulness as internal fluorescence standards, including anthracene, perylene, naphthalene, decacyclene, cobalt(II)-phthalocyanine, copper(II)-phthalocyanine nickel(II)-2,11,20,29-tetra-*tert*-2,3-butyl-naphthalocyanine (all Aldrich), chlorophyllin (sodium-copper salt), fluorescein (sodium salt) and hemin (all Sigma).

Chl *a* and chl *b* standards in acetone have been shown to be stable for several months when stored under inert gas at -20 °C [11,12]. Stability of the prepared zinc-phthalocyanine internal standard solution (IS) was monitored using HPLC, no degradation was observed over a period of 6 months.

2.2. Sample preparation

Spinach, lettuce, iceberg lettuce and endive were purchased in 50–500 g portions at a local supermarket, weighed and stored at -65 °C. For analysis, frozen plant material was crushed into small pieces (ca. 1 cm²) and a 10–20 g aliquot was weighed into a mortar. A mixture of 10 g quartz sand (Merck, fine granular, washed and calcinated) for facilitating cell rupture was added together with 10 g soluble starch (Merck) to increase viscosity. Aliquots of the IS solution containing ~ 0.6 mg IS were added together with 3 ml aqueous phosphate buffer (pH 7, Einecs, Fluka). Mixtures were homogenized thoroughly with a pestle while adding liquid nitrogen repetitively. From the mixtures, 1–4 g were transferred into a 20 ml polyethylene vial containing 2 ml aqueous buffer (pH 7) and 13 ml DMF and soaked for 2 h at -25 °C. After sonification for 5–10 min at 5 °C, the vial content was transferred into a 20 ml disposable syringe filled to a height of 2 cm with quartz sand and filtered through a 0.45 μ m disposable filter (Chromafil A-45/25, Macherey-Nagel, Oensingen, Switzerland). DMF (5 ml) was passed three times through the syringe containing the sample. Fractions were combined in a 50 ml argon flushed glass vial containing 3 ml aqueous buffer (pH 7). The whole preparation was carried out in an ice-bath under dim light.

Collected extracts were further purified for HPLC analysis by solid phase extraction (SPE). SPE columns (Supelclean LC-18 SPE Tube 1 g, Supelco, Buchs, Switzerland) were preconditioned with 2 ml methanol (Merck) followed by 5 ml aqueous phosphate buffer (pH 7). A 1–10 ml aliquot of the pigment extract was diluted with the same volume

of aqueous phosphate buffer (pH 7) for increasing the solvent's polarity. The column was washed after sample loading with 5 ml aqueous phosphate buffer (pH 7) and 6 ml of a methanol/phosphate buffer mixture (94:6, v/v). Pigments and IS were eluted with 6 ml pure methanol followed by 4 ml DMF at a flow rate of ~ 1 ml/min, allowing the resin to run dry at the end. The methanol and the DMF fractions were combined in argon flushed polyethylene vials and stored immediately at -25°C for HPLC analysis. SPE columns were regenerated with acetone (5–10 ml) and reused up to five times. Prepared samples can be stored for later HPLC analysis for several weeks at -25°C . Chlorophyll solutions in DMF have been shown to be stable for at least 20 days at 5°C [19,20].

2.3. HPLC analysis

Samples were analyzed by reverse-phase HPLC using fluorescence detection. The HPLC system consisted of an injection port (Rheodyne 7125) with a $5\ \mu\text{l}$ loop (Rheodyne, Cotati, CA, USA), a high pressure pump (Bischoff Model 2200, Bischoff, Leonberg, Germany), a degasser (ERC 3511, Erma, Tokyo, Japan), a gradient/system control mixer (300 Benchtop, Autochrom, Milford, MA, USA), a fluorescence detector (RF 511, Shimadzu, Duisburg, Germany), a column oven (CTO 10 AC, Shimadzu) and an integrator (Merck-Hitachi D 2520 GPC, Merck). A 25 cm narrow bore RP-18 column (Merck supersphere, LiChroCART 250-2, $4\ \mu\text{m}$ particles, 2 mm inner diameter) was used in combination with a 4 mm RP-18 (LiChroCART 4-4, filled with LiChrospher 100, $5\ \mu\text{m}$ particles, Merck) pre-column. Flow rate was adjusted to 0.28 ml/min and temperature was set to 31°C . Chlorophylls and their C-10 epimers were eluted in 100% methanol, methanol was replaced after 9.5 min by a mixture of 80% methanol, 15% acetone and 5% DMF within 6.5 min using a linear gradient. IS, pheophytins and their epimers were eluted from the column in the same eluent. The column was conditioned for the next run by changing the eluent back to 100% methanol within 2 min. Excitation/emission wavelengths for fluorescence detection (in nm) were 429/664 for chl *a* and chl *a'*, 456/648 for chl *b* and chl *b'*, 405/661 for phe *a* and phe *a'*, 436/655 for phe *b* and phe *b'* and 360/665 for zinc-phthalocyanine. Chlorophylls and pheophytins were identified by their retention times as determined for the

prepared standard solutions. Epimers were identified by comparison with chromatograms reported in the literature and excitation/emission characteristics [21–24].

2.4. Statistics and calculation

Calculations were done using commercial spreadsheet software (Excel 97, Microsoft, Chicago, IL, USA and SPSS 10.0, SPSS, Inc., Chicago, IL, USA). Means are expressed as arithmetic means ± 1 S.D. *P*-values < 0.05 were referred to as statistically significant.

Prior to quantification of chl *a* and chl *b* in vegetable foods, the instrumental response factors (RF) for chl *a* and chl *b* against the IS had to be determined based on external calibration methods. Based on these response factors, chl *a* and chl *b* in the plant foods can be quantified based on the measured areas of the pigments and the amount of IS added prior to sample preparation.

$$\text{RF} = \frac{A_{\text{IS}} C_{\text{chl } a, \text{chl } b}}{C_{\text{IS}} A_{\text{chl } a, \text{chl } b}}$$

with A_{IS} and $A_{\text{chl } a, \text{chl } b}$ being the areas of the peaks of IS and chl *a* or chl *b* in the chromatograms and C_{IS} and $C_{\text{chl } a, \text{chl } b}$ the corresponding concentrations of the external standard solutions, similar as described previously [25]. Pearson correlation coefficients *R* as indicators for linearity were calculated for the regression curves of each pigment.

2.5. Stability of response factors and recoveries

To make use of the zinc-phthalocyanine signal for quantification, the response factor, i.e. the relative fluorescence detector response for a chlorophyll compound versus the IS, has to be independent from the respective amount ratio. To verify whether response factors were constant, varying amounts of chl *a* and chl *b* (0.15–3.2 μg per compound) were added to constant amounts of IS (0.3, 0.6 and 0.9 μg), using acetone as the solvent.

To evaluate the long-term stability of the response factors, external calibration curves (Table 1) for IS and pigments were established over a period of 6 months. These calibration curves were, in addition to the pigment mixtures described above, used to calculate response factors based on the slope of the regression curves obtained.

Table 1
Analytical parameters of regression curves for chl *a*, chl *b*, and IS (zinc-phthalocyanine)

	chl <i>a</i>	chl <i>b</i>	IS
Intercept \pm S.D. (AU) ^a	-1.15 ± 1.28	-0.26 ± 0.22	-0.20 ± 0.33
Slope \pm S.D. (AU/CU) ^b	44.24 ± 0.62	18.30 ± 0.17	64.56 ± 1.16
<i>R</i>	0.9997	0.9998	0.9993

Each calibration graph consisted of six measured concentrations of the pigments. Concentration ranges were 0.12–3.41 $\mu\text{g/g}$ for chl *a*, 0.05–2.11 $\mu\text{g/g}$ for chl *b*, and 0.03–0.45 $\mu\text{g/g}$ for IS.

^a AU: area units.

^b CU: concentration units ($\mu\text{g/g}$).

Independently, the amount ratio of IS and chlorophylls and, therefore, response factors should not be altered during sample preparation and analysis. Discrimination of pigments against IS and vice versa translates automatically into a systematic error in the calculated chlorophyll concentration. To evaluate the effect of sample preparation on the response factors, mixtures ($n = 14$) of chl *a*, chl *b* and IS containing chl *a* (6.1–31.8 μg), chl *b* (4.9–27.6 μg) and zinc–phthalocyanine (15.2–35.6 μg) in different amount ratios were prepared from the standards. Mixtures were treated identically to vegetable samples after addition of fresh onion (10 g) at the homogenization step which served as a chlorophyll-free matrix. The percent recovery of the pigments and amount ratios before and after sample preparation and analysis were determined by external calibration.

In addition, recoveries of the pigments were also determined without matrix for the SPE procedure alone ($n = 9$ independent runs), using solutions containing 41.5 μg chl *a*, 15.0 μg chl *b* and 13.9 μg IS. The recoveries were determined based on area units measured before and after SPE.

3. Results

Typical chromatograms obtained for a leafy vegetable (spinach) are shown in Fig. 2. Samples prepared as described showed no or only minor signs of chlorophyll degradation. Chlorophyll degradation became visible when the same extract (10 ml) was acidified with 50 μl 2 M HCl and exposed for 15 h to air and light (Fig. 2). Chlorophylls were found to be degraded into pheophytins (phe *a* and phe *b*) as well as into their C-10 epimers (chl *a'*, chl *b'*, phe *a'*

and phe *b'*). In the degraded sample, a slight tailing of the zinc–phthalocyanine peak was observed. Degradation of chlorophylls alone resulted in no detectable peak formation at retention times close to zinc–phthalocyanine. This points to a slight degradation of the internal standard under the relatively harsh conditions used in this particular experiment. No tailing was observed under the conditions used for pigment analysis in vegetables. Fig. 2 shows that the zinc–phthalocyanine signal can be clearly resolved from the pigment signals even for samples containing multiple degradation products. However, chlorophylls and their epimerization products could not be completely resolved. At the lowest column temperature tested (16 $^{\circ}\text{C}$), chlorophylls and epimerization products could be separated completely, but separation of zinc–phthalocyanine and phe *b* was unsatisfactory.

HPLC analysis of the different mixtures of IS and chl *a* and chl *b* used to investigate the stability of the response factors showed no dependence on the amount ratio of chlorophylls to IS over a range of 0.2–10 (Fig. 3). Average instrumental response factors as determined for the different mixtures (± 1 , S.D.) were 1.39 ± 0.10 for chl *a* ($n = 18$) and 3.53 ± 0.20 for chl *b* ($n = 18$), respectively. In addition, response factors were obtained by dividing the slope of the calibration lines for chl *a* and chl *b*, respectively, through the slope of the IS calibration line, obtained within a period of 205 days. No drift in the response factors with time was observed. Average response factors were 1.46 ± 0.08 for chl *a* and 3.54 ± 0.26 for chl *b* ($n = 4$, respectively), which is not statistically different from the response factors obtained from the mixtures of IS, chl *a* and chl *b* (1.39 ± 0.10 and 3.53 ± 0.20 , respectively; paired Student's

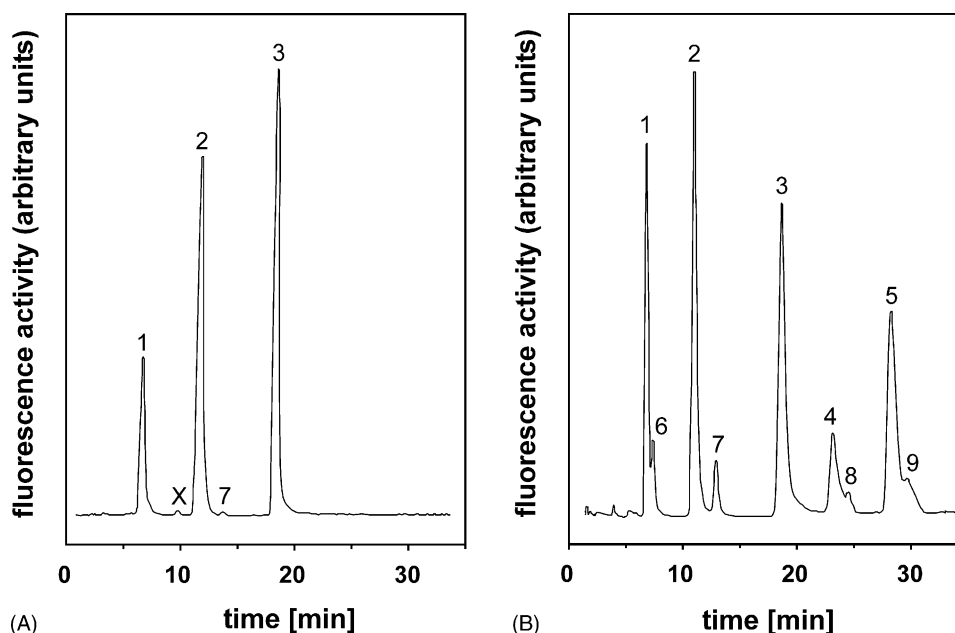


Fig. 2. Chromatogram of spinach, stored frozen until analysis by HPLC (A) and after acidifying the same pigment extract with 0.2 ml M HCl per 10 ml extract and exposure to air and light for 15 h at 20 $^{\circ}\text{C}$ (B). Zinc–phthalocyanine was used as an internal standard (IS). (1) Chlorophyll *b*; (2) chlorophyll *a*; (x) unknown degradation product; (3) IS; (4) pheophytin *b*, (5) pheophytin *a*; (6) chlorophyll *b'*; (7) chlorophyll *a'*; (8) pheophytin *b'*; (9) pheophytin *a'*.

Table 2
Chlorophyll content of some leafy vegetables

Vegetable	<i>n</i>	chl <i>a</i> (μg/g)	chl <i>b</i> (μg/g)	Total chlorophyll (μg/g)	Ratio chl <i>a</i> :chl <i>b</i>
Spinach	5	691 ± 20	193 ± 7	884 ± 18	3.60 ± 0.21
Endive	5	273 ± 26	83 ± 7	356 ± 33	3.29 ± 0.08
Lettuce	3	283 ± 18	70 ± 5	353 ± 24	4.04 ± 0.06
Ice-berg	3	19 ± 1	4 ± 1	22 ± 1	5.07 ± 0.09

Chlorophyll content (±S.D.) of four fresh and immediately frozen green leafy vegetables. Analyses have been carried out using zinc-phthalocyanine as an internal standard.

t-test). Furthermore, RF based on external calibration graphs (*n* = 6) based on pigment solutions stored at −25 °C under argon were stable during a period of 6 months.

The amount ratio of the pigment standards after analysis relative to the amount ratio before analysis was 0.91 ± 0.10 for chl *a*/IS and 0.98 ± 0.12 for chl *b*/IS. Mean recoveries were 88 ± 9% for chl *a*, 95 ± 13% for chl *b* and 98 ± 8% for zinc-phthalocyanine for the different mixtures. Recoveries differed not significantly between chl *b* and IS (paired Student's *t*-test) but recovery of chl *a* was significantly lower when compared to zinc-phthalocyanine (*P* < 0.005) and chl *b* (*P* = 0.01). No discrimination of chl *a* against IS was observed when mixtures were analyzed without the preceding extraction step and without addition of fresh onion. Recoveries for the SPE/HPLC procedure (extraction efficiencies) alone were 98 ± 5, 94 ± 2, and 99 ± 6% for chl *a*, chl *b* and IS, respectively. Recoveries for phe *a* and phe *b*, respectively, were 95 ± 13, and 92 ± 10%, which shows that the method can be used basically to monitor chlorophyll degradation too.

Instrumental precision of the HPLC system was evaluated using two standard solutions containing chl *a* and chl *b* at different amount ratios relative to IS. Solutions were injected consecutively (*n* = 5). Relative S.D. values were 4.1% (~6.5 μg/g) and 3.2% (~1.0 μg/g) for IS, 2.1% (~4.5 μg/g) and 3.2% (~0.8 μg/g) for chl *a* and 4.8% (~3.0 μg/g) and

3.8% (~0.5 μg/g) for chl *b*. Detection limits were estimated by the USA EPA approach [26], using replicate analysis (*n* = 6) of a highly diluted chlorophyll standard containing 98 ng/g chl *a*, 47 ng/g chl *b* and 28 ng/g IS, respectively. Detection limits defined as three times the S.D. obtained for the analysis of the diluted standards were 6.6 ng/g for chl *a*, 10.7 ng/g for chl *b* and 4.5 ng/g for zinc-phthalocyanine.

Chlorophyll contents of the different vegetables were determined in a series of independent runs and are shown in Table 2. The total chlorophyll content and the chl *a*:chl *b* ratio varied between vegetables. Iceberg lettuce had the lowest total chlorophyll content but the highest chl *a*:chl *b* ratio. In the other vegetables analyzed, the chl *a*:chl *b* ratio was found to be similar. Mean recovery of IS over all runs (*n* = 16) was (100 ± 12)% as determined by external calibration. For independent analysis of the same vegetable, the relative S.D. varied between 2.0% for spinach to 9.3% for endive.

4. Discussion

Zinc-phthalocyanine was found to be sufficiently stable, could be separated well from chlorophylls and degradation products by HPLC, showed similar fluorescence properties compared to chlorophylls and can be purchased in sufficient purity at a relatively low price. Zinc-phthalocyanine would thus appear to be the most suitable internal standard for chlorophyll analysis of the different compounds which have been evaluated in the present study. Polycyclic aromatic hydrocarbons (anthracene, perylene, naphthalene, decacyclene) differ strongly from chlorophylls in chemical and fluorescence properties, chlorophyllin and fluorescein are too polar, and hemin and nickel-(II)-2,11,20,29-tetra-*tert*-2,3-butyl-naphthalocyanine are only poorly soluble in the most suitable solvents (DMF, methanol and acetone) used for chlorophyll extraction. Other investigated phthalocyanines (cobalt-(II)-phthalocyanine and copper-(II)-phthalocyanine) were found to co-elute with the pheophytins.

Column temperature was found to be decisive to achieve complete pigment separation. Separation of the zinc-phthalocyanine signal became possible by increasing the temperature to 31 °C. Separation of chlorophyll and pheophytin on the HPLC column from their C-10 epimers was less good under these conditions compared to lower temperatures but could be achieved, in principle, by using a

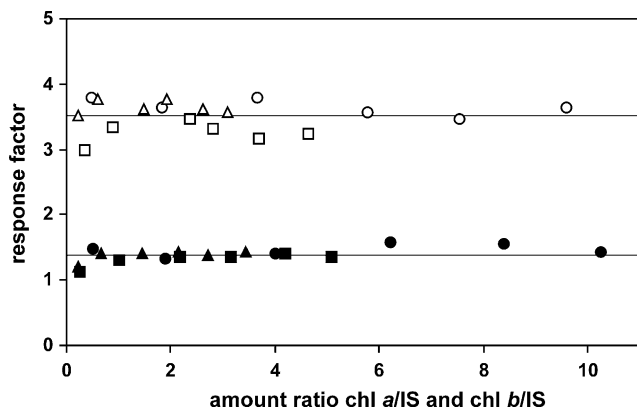


Fig. 3. Response factors of chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) as determined for mixtures containing chl *a* and chl *b*, respectively, in different amount ratios to zinc-phthalocyanine as the internal standard (IS). Mixtures of chl *a* and IS varied in zinc-phthalocyanine concentration: 0.3 μg/g (●), 0.6 μg/g (■) and 0.9 μg/g (▲). Mixtures of chl *b* and IS varied in zinc-phthalocyanine concentration: 0.3 μg/g (○), 0.6 μg/g (□) and 0.9 μg/g (△). The dashed lines represent the two means.

methanol/water mixture for column elution before switching to pure methanol. This was not possible with our equipment which allowed to run binary gradients only.

To separate the pigments from the plant matrix for HPLC analysis, DMF extraction of the homogenized plant material was used in combination with SPE purification. DMF allows chlorophyll extraction from plant matrices at high extraction yields [19,27]. SPE was chosen because it allowed reproducible processing of large sample numbers and pigment enrichment and reduces consumption of chemicals.

Despite the precautions taken during sample preparation to prevent chlorophyll degradation, chl *a* recovery for the entire sample preparation procedure was lower compared to chl *b*. Because recoveries determined for the SPE procedure alone indicated no significant losses, discrimination of chl *a* against chl *b* during DMF extraction or degradation during sample preparation prior to SPE are the most probable explanations. It has been shown in the past that chl *a* is more rapidly degraded than chl *b* to the corresponding pheophytins at lower pH [28,29]. However, the observed chl *a* losses during sample preparation were in the order of 10%, which is not considered a major limitation for most applications. If required, empirically derived correction factors could be introduced to correct for chl *a* underestimation.

Data obtained for the four analyzed vegetables agreed well with literature data. Chlorophyll concentration is known to vary significantly between plants. Chlorophyll contents as high as 1 mg/g can be found in spinach and is less than 0.1 mg/g in broccoli and brussels sprouts [22,30]. As expected, spinach had the highest chlorophyll content of the vegetables analyzed (691 µg/g), the lowest chlorophyll content was found in iceberg lettuce (19 µg/g). The amount ratio of chl *a*:chl *b* is also known to vary between vegetables. Typical chl *a*:chl *b* ratios are in the range of 2.8–4.7 [22,30] which is in good agreement with our results. Precision in chlorophyll analysis differed for independent runs of the same vegetable, ranging from 2.0% for spinach to 9.3% for lettuce. These differences in reproducibility cannot be explained by the lower chlorophyll content alone. Sampling also seems to limit the achievable reproducibility. Plant material consists of stems, stalks and leafy parts which usually differ significantly in chlorophyll content. Multiple sampling of the same plant leads, accordingly, to stronger variations in the data. This suggests that sampling strategies have to be evaluated carefully to make fully use of the potential of zinc–phthalocyanine as an internal standard for chlorophyll analysis.

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