Improvements in the measurement of chlorophylloids in soybean oil

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Abstract

An improved procedure using HPLC with fluorescence detection for the measurement of chlorophylloids in refined bleached soybean oil was developed. These improvements allow a simple and rapid method for the analysis of chlorophylloids in products including chlorophyll, pheophytin and pyropheophytin in refined bleached soybean oil. By exchanging the standard photomultiplier tube in the fluorescence detector with a red-sensitive Hamamatsu R928HA PMT, a 300 fold increase in sensitivity was achieved. A direct analysis of diluted soybean oil was performed without further sample preparation. Calibration of the analysis was performed by quantification of purified chlorophylloids by UV/VIS spectroscopy.

Introduction

In measuring the quality of Soybean oil, the analysis of chlorophyll degradation products is an important factor. Chlorophylloids provide the majority of color compounds in soybean oil. In this work, chlorophylloids were measured in refined and bleached soybean oils to provide a better understanding of the performance of different bleaching clays with typical and difficult to bleach oils.

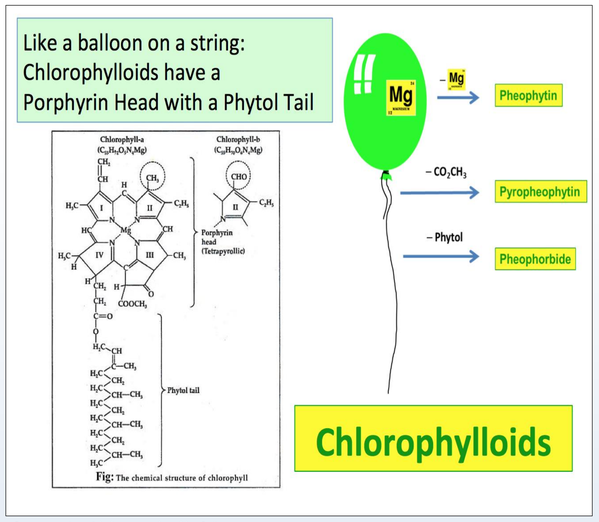

Figure 1: Chlorophylloids

The primary degradation products of chlorophyll are Pheophytins, Pyropheophytins and Pheophorbides. Chlorophylloids are normally measured by the AOCS Cc 13d-55 (Lovibond) method or by the AOCS Ch 4-91 Vis method. These methods relate the measured color to a "chlorophyll concentration". These methods provide limited information on the degradation products present. And as discussed in our previous paper underestimate the total chlorophylloids ([3]).

Historically, most HPLC work has been done by fluorescence or UV/VIS detection sometimes using SPE for sample concentration. In this study, HPLC with florescence detection is used to quantify the individual degradation products. By upgrading our detector, all samples are able to be tested by 1:5 dilution in acetone followed by HPLC-FL.

Properties of some of the oils measured in study 1 are summarized in Table 1.

Table 1. Typical vs Atypical RSBO Characteristics

Oil Type	Starting Oil Levels: Chlorophyll ppb AOCS	Starting Oil Levels: ppm Trace Elements	Common Knowledge: Chlorophyll ppb AOCS	Common Knowledge: ppm Trace Elements	% Clay Used To Achieve Specs: Reported	% Clay Used To Achieve Specs: In Test
Typical	200 - 500	0.1 - 13.5	< 1500	< 10	< 1.5%	0.2 - 0.4
Atypical (DTB)	2000 - 3500	2.1 -14.3	> 500	>10 ppm, high NHP	> 1.5%	2.0 - 3.5

Note: DTB = Difficult to Bleach

Materials and Methods

Materials

- Refined soybean oils (RSBO; 2 US, 2 foreign)
- ACS grade citric, phosphoric and hydrochloric acids
- ACS grade Pyridine — Sigma Chemical Co. (St. Louis, MO)
- HPLC grade methanol, tetrahydrofuran, acetone and water
- Natural clay (pH 8.2) and acid activated clay (pH 3.3); (USA, Oil-Dri)
- Spinach — Store purchased
- Chlorophyll a — Sigma Chemical Co. (St. Louis, MO).
- Chlorophyll b — Indofine chemical Co. (Hillsborough, NJ)
- Discovery DSC-18 3ml-500mg SPE tubes 52603-U — Supelco
- Unison 3 µM UK-C18 (250 X 4.6) ODS (Imtakt USA, Portland, OR)
- Kinetex 5 µM C18 100Å (250 X 4.6) 00G-4601-E0 (Phenomenex USA)

Bleaching Procedure

RSBO (200g) taken to 110°C for 30 min w/100 mm Hg vacuum varied conditions: w/wo 500 ppm citric acid, 0.4% added water, with a pre-determined dosage of clay at which "specs" for deodorized red color, chlorophyll and trace elements were achieved.

Standard Preparation

- Pheophytin (Phy) a & b were prepared from chlorophyll a & b by reaction w/ HCl ([1]).
- Pheophorbide (Pho) a & b were prepared from chlorophyll a and b by reaction w/H_3PO_4 ([2]).
- Pyropheophytin (Pry) a & b were prepared from Pheophytin a & b by refluxing in Pyridine at 110° C for 24 hours ([2]).
- All the prepared standards were purified using SPE.

HPLC Methods

The analysis was performed using an Agilent 1260 Infinity Series HPLC system with a G1311B 1260 Quaternary Pump, G1329B 1260 Autosampler, G4212B 1260 DAD and G1321C 1260 FLD.

Preliminary work with the measurement of refined soybean oils and spinach extracts found that the FLD did not have adequate sensitivity for this study @ 670 nm without performing extensive SPE sample preparation.

To improve the sensitivity of the analysis we replaced the standard blue sensitive photomultiplier tube, Hamamatsu R212HA , that is supplied by Agilent with a more red sensitive tube, Hamamatsu R928HA ([4]). Comparison of samples measured before and after the replacement found an average improvement of 32,600%. Figure 2 shows the comparison of these 2 photomultiplier tubes.

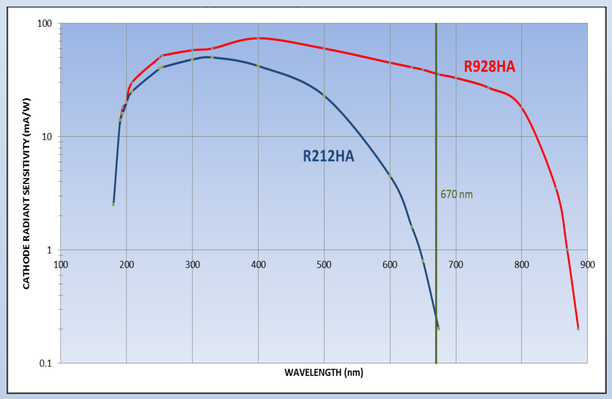

Figure 2: Typical Photomultiplier Tube Spectral Response

HPLC Study 1 conditions

With the improved detector response, a preliminary study was performed with our existing Column.

Column: Unison UK-C18 (250 X 4.6) ODS 3 µM
Injection volume: 10 µL
Detection: FLD, excitation 430 nm/emission 670 nm
Mobile phase:

Time (min)	%A Water	%B Tetrahydrofuran	%C Methanol	%D Acetone	Flow rate (mL/min)
0	4	0	67	29	1.4
5	4	0	59	37	1.4
30	4	0	59	37	1.4
30.1	0	100	0	0	1.4
34	0	100	0	0	1.4
34.1	4	0	67	29	1.4
36.1	End of test, ready to inject new sample				

HPLC Study 2 conditions

Based on the results of study 1, the chromatographic method was improved to provide better results and a designed experiment was conducted.

Column: Kinetex 5 µM C18 100Å (250 X 4.6)
Injection volume: 5 µL
Detection: FLD, excitation 430 nm/emission 670 nm
Mobile phase:

Time (min)	%A Water	%B Tetrahydrofuran	%C Methanol	%D Acetone	Flow rate (mL/min)
0	4	0	86	10	1.5
3	4	0	86	10	1.5
9	4	0	62	34	1.5
30	4	0	62	34	1.5
40	0	60	0	40	1.5
45	0	60	0	40	1.5
45.1	4	0	86	10	1.5
46.1	End of test, ready to inject new sample				

Results and Discussion

From freshly purchased Chlorophyll a and b standards, all the chlorophylloid compounds were manufactured in ~100 µg quantities, purified though repeated SPE procedures to >90% purity and dissolved in Acetone. By dilution and measurement by UV, Lovibond, and HPLC, calibration curves for each chlorophylloid were prepared as shown in graphs below: concentrations were determined by UV using published absorptivities ([2]).

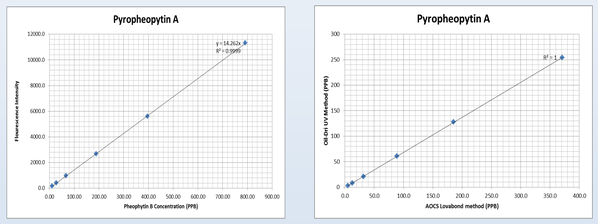

Figure 3a & b: Calibration Curves

These calibrations were used to convert the fluorescence intensities to ppb concentrations. Below is a comparison of typical and atypical oils. The Unknown peak includes highly retained unknown color compounds removed during column cleanup.

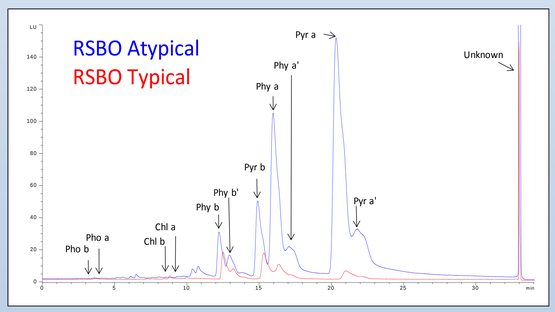

Figure 4: Study 1 Oil Comparison

After optimizing the new method, for the compounds of interest, complete separation was achieved for all peaks except pyropheophytin b and pheophytin a, with a method detection limit of <2 ppb for all compounds. Improved separation of the highly retained compounds was achieved. Below is a comparison of an unbleached oil and a bleach run of it. A 6 clay 4 oil designed experiment is in process to expand on the conclusions from Study 1.

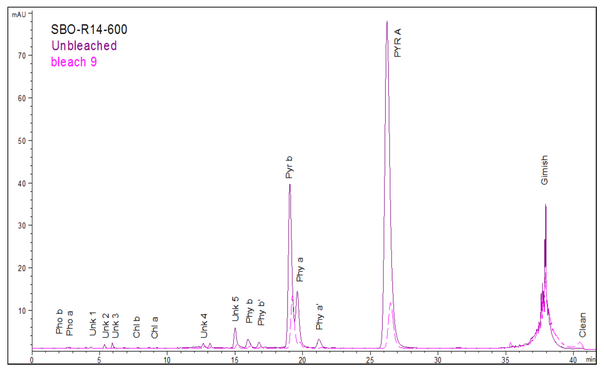

Figure 5: Study 2 bleaching result

Conclusions

- No chlorophyll a or b analogues were found in starting oil samples.
- Linear correlation between HPLC total chlorophylloid assay vs–
  - AOCS assay (Y= 0.67X – 1.2; R^2=0.969); n=64 pairs.
  - AOCS adjusted assay (Y= 0.73X +38; R^2=0.972); n=64 pairs
- By HPLC total chlorophylloid assay method
  - Citric (aq) addition improved bleaching performance across both clay and oil types (45% average reduction).
  - Citric (aq) addition had the greatest effect on the bleaching performance of the natural clay (54% vs 35% average reduction)
- Clay demand was mostly driven by concentration of total chlorophylloid and not by specific chlorophylloid; i.e. DTB atypical chlorophyll is one order of magnitude greater, therefore clay demand went up by a comparable order of magnitude.

Next Steps

- The results of study 2 will be published later this year.
- Some of the unknown peaks have been observed in prior works without being identified([1,2,4]). Once the identities of these compounds can be confirmed by HPLC-MS, the absorptivities will need to be measured to quantify these color compounds.
- Currently the absorptivities of closely related compounds, for example Phy a and Phy a' , are only known collectively. The determination of the absorptivity coefficient for each isomer separately would improve the quantification.

References

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3. D.D. Brooks, and A. Litin, Adsorptive performance of bleaching clays in soybean oils based on adsorption of chlorophylloid analogs and deodorized oil color, 106th AOCS Annual Meeting; (2015).
4. X.Li, S. Wang and M. Woodman, Agilent Application Note 5991-4384EN (2014).