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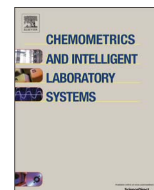

A multiphase experiment for the analysis of bioactive compounds in canola oil: Sources of error from field and laboratory

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ABSTRACT

Multiphase designs are an effective technique to assess all sources of variation in an experiment; however they have not yet been widely applied to analytical chemistry. In this study a multiphase design was used to evaluate sources of error from the field and laboratory phases of an experiment involving canola growing, oil extraction, sample preparation, and analysis using high performance liquid chromatography coupled to diode array detection and tandem mass spectrometry (HPLC-DAD-MS/MS). Several classes of bioactive compounds – tocopherols, carotenoids and sterols – were measured in the canola (*Brassica* napus) oil from 64 different genotypes to be assessed for varietal and environmental influence. Other factors which might contribute to error were identified, incorporated into the design, and their associated error calculated and accounted for. The field and HPLC phases were the largest contributors to total error, with only small influences from the extraction and preparation phases. Likelihood ratio testing of the nested multiphase models proved that high precision was achieved by use of the multiphase design, and identified possible improvements for future laboratory work. In the first reported application of a multiphase design to multi-stage laboratory analyses, the design was shown to offer considerable advantages over traditional approaches particularly in reducing total sample number, time and cost of analysis, as well as more comprehensive monitoring of experimental error.

1. Introduction

Evaluating and minimising error in laboratory analyses is critical. Without this, estimations of the uncertainty in the data are impossible to determine correctly. There is a high expectation from the scientific community for the generation of reliable experimental outcomes using robust experimental designs and statistical analyses. However, an analysis is typically dependent on the type of data obtained and thus there is no 'standard' statistical analysis. Moreover, there is no uniform method to evaluate error. As a result, statistical tests are often applied incorrectly, with the selected test not always the best fit for the data [1]. Although there have been several reviews addressing these concerns, there remain many situations where incorrect estimation and evaluation of experimental error occur [1–4].

Another important concept in the evaluation of experimental data is that of precision, yet it has been reported that there is often "incorrect estimation and inappropriate use of precision" [3]. In the simplest of definitions, precision is the 'closeness of measurements compared to one another'. As an ordinal quantity, there is no direct way to measure precision, however, there are many ways to assess and indirectly measure precision, the most common of which is standard deviation. The correct evaluation of experimental precision not only relies on correct calculations, but also on reliable laboratory practice. The link between precision and error is provided in Thompson's concept of the "ladder of errors" [3], which shows how experimental values may become less precise through the error inherent in each stage of an analytical procedure.

Comprehensively assessing error in an analysis requires full replica-

Abbreviations: HPLC-DAD-MS/MS, High Performance Liquid Chromatography coupled to diode array detection and tandem mass spectrometry; APCI, Atmospheric Pressure Chemical Ionization; MRM, Multiple Reaction Monitoring; m/z, mass to charge; REML, restricted estimate maximum likelihood; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation

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tion of every stage of the analysis from sampling through to the final measurement of analyte concentration *via* detector response. Full replication at each stage of the laboratory process typically results in the number of samples becoming too large to be practical, therefore it is common for chemists to select only particular stages in the experiment to perform replication, without prior investigation [3]. They often simply discount the other sources of error that could occur during an experiment, for example during sample storage, extraction, or preparation, leading to underestimation of the error. However, it is of great importance to first determine at which stage in an analytical determination the largest variance occurs, and what the largest sources of variance are within each stage. This information may then be used to justify which stages require additional replication and which stages can be ignored in future analysis, with reasonable safety, and with little effect on the precision of the results. Such an analysis of experimental error can be provided through the application of multiphase design methodology [5].

Multiphase designs have been widely applied in plant biology and field experiments, though their potential for use in analytical chemistry is just emerging. These designs rely on partial replication and re-randomisation at two or more 'phases' in an experiment in order to estimate the contribution of errors at each stage to the overall variance ('ladder of errors') [1,6–8]. By monitoring the key factors in each phase, it is possible to determine which experimental variables cause the largest error. In this context, phase refers to the 'stage' of the analytical process or experiment and not to physical state (e.g. solid, liquid, gas). To remain consistent with the original terminology of 'multiphase' designs, the term 'phase' is used here to define the stage of the analytical process. Multiphase designs have been used successfully in plant pathology and biology field experiments for measuring non-genetic sources of variation [7,8]. The application of field multiphase designs consisting of an additional laboratory phase has been suggested, in which the importance of incorporating controls and re-randomisation in all phases was emphasised [6]. In the same paper, two-phase experiments consisting of one field and one laboratory phase, as well as those involving several field phases were discussed. However, these designs have not yet been widely applied in analytical chemistry. Moreover, to the authors' knowledge, a multiphase design has not yet been applied to monitor error in an experiment consisting of field and multiple laboratory phases.

In this study a multiphase experimental design was applied to the sampling, preparation and quantification of three classes of bioactive compounds in canola oil from field-grown seed samples. Sterols, tocopherols and carotenoids are classes of so-called 'minor' compounds that are present in low concentrations in canola oil, but nevertheless may be of considerable health benefit to consumers. Research to maximise their retention in commercially-processed oil is gaining interest [9]. The most abundant sterols in canola oil, β-sitosterol, campesterol and brassicasterol, were examined in their free and esterified forms along with the main tocopherols, α-, γ- and δ-tocopherol, and carotenoids, β-carotene and lutein. The experiment consisted of a field phase, two sample preparation phases, and one HPLC analysis phase. Within each phase, a number of factors were built into the design to determine the largest sources of experimental error. Partial replication (by the addition of a number of samples as replicates at the beginning of each phase) was applied, and the variance between replicates used to estimate error. The replication rates were determined to allow for error estimation whilst keeping total sample numbers managable. The study was predicted to reveal the factors and stages of the experiment that invoked the highest error, and provide a comprehensive assessment of the distribution of error across all experimental phases. Additionally, by monitoring several analytes using two detectors, further precision testing could provide an indication of the reliability of each detector, and present novel and efficient ways to further assess and validate an experimental procedure.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade 95% n-hexane (Scharlau) was purchased from Chem Supply (Gillman, SA) and analytical grade ethyl acetate was purchased from Sigma-Aldrich (Castle Hill, NSW). β-carotene (purity ≥ 97%), α-tocopherol (purity ≥ 96%), γ-tocopherol (purity ≥ 96%), δ-tocopherol (purity ≥ 90%), β-sitosterol (analytical standard), campesterol (analytical standard), brassicasterol (analytical standard) and cholesterol (analytical standard) were all purchased from Sigma-Aldrich (Castle Hill, NSW). Lutein (purity ≥ 95%) was purchased from Extrasynthese (Genay, France).

2.2. HPLC analysis

Analysis was conducted according to the methodology previously described [10]. In brief, a Varian Star 9010 binary pump was used with an Agilent 1200 series High performance liquid chromatography coupled to a diode array detector and tandem mass spectrometer (HPLC-DAD-MS/MS). Normal Phase chromatography was conducted using a Phenomenex luna silica column (150 mm × 4.6 mm, 3 µm). Gradient elution was performed with n-hexane and ethyl acetate [10]. The MS was run in positive Atmospheric Pressure Chemical Ionization (APCI) mode using Multiple Reaction Monitoring (MRM) mode. Ion transitions for the compounds analysed were: mass to charge (m/z) 551→119.2 (lutein), 537.5→119.2 (β-carotene), 430.0→165.0 (α-tocopherol), 416.0→151.0 (γ-tocopherol), 402.0→137.0 (δ-tocopherol), 397.4→257.3 (β-sitosterol), 383.4→161.3 (campesterol), and 381.4 → 297.3 (brassicasterol). Wavelengths of 454 nm for carotenoids, and 294 nm for tocopherols were monitored using DAD. The concentrations of two carotenoids (β-carotene and lutein), three tocopherols (α-tocopherol, γ-tocopherol and δ-tocopherol) and three phytosterols (β-sitosterol, campesterol and brassicasterol, free and esterified forms) were measured. An internal standard, cholesterol, was quantified using m/z 369.4→161.0. Additionally, the two most abundant tocopherols, α-tocopherol and γ-tocopherol were measured using two detection techniques (DAD and MS). These were treated as separate analytes (i.e. α-tocopherol DAD, α-tocopherol MS) during statistical analysis of the data to allow for comparison of detector response.

2.3. Plant materials and field design

In 2013, 64 canola genotypes were grown in two field trials using sites at Wagga Wagga, New South Wales and Westmere, Victoria (−37.688920 °S, 142.969334 °E). The experiments consisted of balanced partial replication of the genotypes, with 1.5 replicates at each site and three replicates overall. The Wagga Wagga plots were arranged in a 6 × 16 grid, the Westmere plots in a 4 × 24 grid. Plot sizes were 10 m × 1.5 m with 8 lines per plot. The experiments were sown on 7th May 2013 and harvested on 18th December 2013. Climate data for both sites were similar in terms of temperature range, however there was considerably higher rainfall at Westmere, particularly during the times of maturing and harvesting.

After machine harvest, the seed was stored in large bags at room temperature in the dark for 3 months, after which a subsample of the seed (~500 g per plot) was cleaned using an Aerovac aspirator, placed in sealed plastic containers with desiccant, and stored at 4 °C in the dark. A total of four seed samples were not suitable for laboratory analysis because two had suffered from moisture damage with mould occurring in the large seed bags, and two other samples were inadequate in size for processing. Thus, 188 field-sourced seed samples constituted Phase 1 of the multiphase design (Fig. 1).

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