# Alternative Methods for Alternative Proteins

## A Look at the Complexities of Existing Analytical Methods for Novel Plant-based Products

## Abstract

While the current explosion in growth of plant-based food products has been a surprise to some, the inevitability of its presence in consumer markets, has not. With rising concerns about the environmental impact of animal farming, animal welfare, and the nutrition of traditional meat and dairy products, plant-based alternatives are becoming a mainstay to grocery stores, restaurants, and retailers. However, the rapid growth and acceptance by consumers has led to a gap in the abilities of regulators and standards groups to monitor and evaluate the efficacy of current analytical techniques. From in-house proximate analysis and nutritional label testing, to adhering to FDA requirements on the level of contaminants and more, everything associated with analyzing alternative protein products still needs to be formally defined. While ISO TC34, the standards committee for food products that guides CODEX and other global testing requirements, has already launched a working group to develop testing requirements for alternative protein products, most manufacturers are being forced to implement "best fit" methods as a way to adapt quickly, regardless if they truly are the best fit or not. With a little due diligence and a keen scientific eye, manufacturers can be certain they are implementing the right techniques now, avoiding major SOP revisions and keeping both consumers and regulators happy. This whitepaper reviews the analysis of several alternative proteins and challenges associated with their analysis, including how plant-based products are similar to traditional dairy and meat products, where they differ, and what options are available to aid in better testing practices.

## Introduction

The first review will be in the area of proximate analysis, the quantitative determination of macromolecules such as fat or carbohydrates, which are critical test points for consumers and producers alike. Accurate proximate analysis testing has long been a mainstay in traditional dairy production, allowing the best processors to optimize various aspects of their manufacturing, in order to reduce loss and implement least cost formulation, while adhering to the utmost quality standards. These proximate tests can be performed in-house, by an onsite system or quality team, or can be sent to an ISO 17025 certified laboratory for reference testing, receiving results anywhere from 2-7 days depending on the required test and turnaround time (TAT) of the lab. These proximate tests are typically related to the reporting on nutritional labels, although not always, in an attempt to fit quality standards outlined during initial product development. For manufacturers, however, a more critical portion of proximate analysis is the need for accurate and precise testing of moisture/solids, fat/oil, or protein content. While other analyses are required for reporting or minor production limitations, it is typically a combination of tightly controlling one, if not all three, of these components that can lead to the greatest cost savings or loss.

To start, moisture analysis is a simple process that does not have much variability in traditional testing. The existing methods adapt well to new and novel alternative products; whether an oven method for batch drying is used, a halogen or IR moisture balance for results in 10-20 minutes, or microwave/IR drying, like CEM's SMART 6™, for results in 2 minutes, the methods remain the same. However, when you start to look at the more complex techniques for fat and protein testing, a variety of obstacles begin to present themselves.

## Obstacles in Fat Determination

Historically there are two broad approaches for fat analysis with endless methods and technologies: reference extraction methods like Soxhlet, Mojonnier, Rose-Gottlieb, and Babcock, or rapid calibration methods like NIR, FTIR, and NMR. More simply, the reference extraction methods break down into some combination of ether extraction with or without acid/base hydrolysis. Traditional food products have a long history of validation for their defined methods, with plenty of supporting data. However, the nature of plant-based products introduces problems in deciding which technique is accurate, followed by a determination of what level of accuracy and repeatability is achievable and acceptable. This is especially true for dairy products thanks to the complexity of lipid bonds they contain, a thorn in the side of any lipid chemist.

Milk products are some of the few samples that require base hydrolysis, relating to the nature of the milk lipids and how they interact with themselves and other components like sugars (lactose) and proteins. Few other food products use this base hydrolysis method, sometimes referred to as Mojonnier or Rose Gottlieb, relying instead on an acid hydrolysis extraction or simple Soxhlet extraction. The problem that arises with plant-based alternative protein products is identifying the correct extraction technique as to not risk under or over stating the fat content due to under- or over-extraction. As an example, with coconut-based yogurt, do you follow the extraction method for coconut (AOAC 948.22 – Fat in Nuts – Pet Ether Extraction) or the traditional Mojonnier technique for yogurt (AOAC 989.05)? Or acid hydrolysis (AOAC 950.54), the "catch all" for fat extraction of unknown samples? One technique for solving this has been to take the summation of all known fatty acids in the sample, as determined by GC-FID (AOAC 996.06 – FAMEs method), however, this still relies on choosing an acid or base hydrolysis and the wrong one will lead to an incorrect FAMEs result. In **Table 1**, the results from a recent coconut yogurt evaluation investigating various methods are shown. Since a defined method does not exist for the product, the contract laboratories were insistent on the use of AOAC 950.54, acid hydrolysis. However, after a round robin of testing via three different reference methods, it was shown that base hydrolysis (AOAC 989.05) better aligned with FAMEs and the ORACLE, with acid hydrolysis being the clear outlier, seen in **Figure 1**.

**Table 1**. Data Compiled from Round Robin of Coconut-based Yogurt Products

| Sample | ORACLE Result | Method | Lab | Result 1 | Result 2 | Average | St Dev | Method Avg | Method SD |
|---|---|---|---|---|---|---|---|---|---|
| Clarified pre/post | 1.16 | 950.54 | CEM | 1.48 | 1.41 | 1.45 | 0.05 | 1.45 | 0.12 |
| | | 950.54 | Eurofins | 1.44 | 1.46 | 1.45 | 0.01 | | |
| | | 950.54 | Silliker | 1.44 | 1.15 | 1.30 | 0.21 | | |
| | | 989.05 | CEM | 1.13 | 1.18 | 1.16 | 0.04 | 1.12 | 0.04 |
| | | 989.05 | Eurofins | 1.11 | | 1.11 | | | |
| | | 996.06 | Eurofins | 1.20 | | 1.20 | | 1.20 | |
| Unclarified pre/post | 1.45 | 950.54 | CEM | 1.83 | 1.93 | 1.88 | 0.07 | 1.89 | 0.15 |
| | | 950.54 | Eurofins | 1.99 | 2.02 | 2.01 | 0.02 | | |
| | | 950.54 | Silliker | 1.86 | 2.24 | 2.05 | 0.27 | | |
| | | 989.05 | CEM | 1.53 | 1.57 | 1.55 | 0.03 | 1.41 | 0.15 |
| | | 989.05 | Eurofins | 1.29 | | 1.29 | | | |
| | | 996.06 | Eurofins | 1.60 | | 1.60 | | 1.60 | |
| Sample #5 | 1.61 | 950.54 | CEM | 2.14 | 2.07 | 2.11 | 0.05 | 2.02 | 0.12 |
| | | 950.54 | Eurofins | 2.00 | 1.95 | 1.98 | 0.04 | | |
| | | 950.54 | Silliker | 1.91 | 2.21 | 2.06 | 0.21 | | |
| | | 989.05 | CEM | 1.64 | 1.61 | 1.63 | 0.02 | 1.57 | 0.07 |
| | | 989.05 | Eurofins | 1.50 | | 1.50 | | | |
| | | 996.06 | Eurofins | 1.58 | | 1.58 | | 1.58 | |

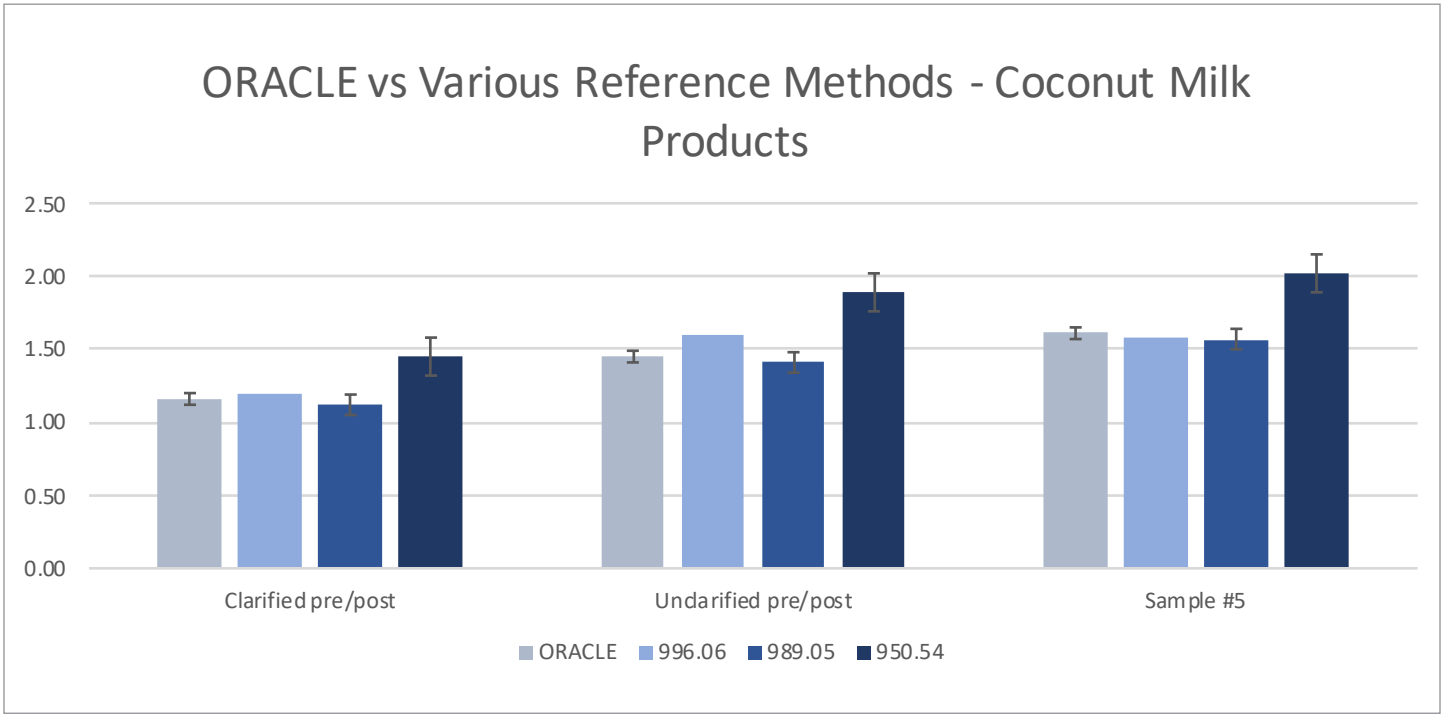


**Figure 1**. Comparison of average results of the methods used for testing coconut-based yogurt products

While these issues are primarily found in the choice of reference extraction method, they are only compounded with most rapid methods. Reference testing errors are amplified with NIR technology, and historical data required for building a strong calibration library, doesn't yet exist. CEM's new NMR Fat Analyzer, the ORACLE, is able to overcome and improve upon both NIR technology and extraction method determination.

The ORACLE is not reliant on calibrations or reference methods, it is a universal fat analyzer that detects lipid molecules, regardless of fat source or sample matrix. This means that new plant-based products, like those listed in **Table 2** and even those still in R&D whose composition is changed constantly, can be accurately analyzed without the need to perform any reference testing.

**Table 2**. Some of the various plant-based sources by product types successfully analyzed by ORACLE

| | Raw Materials | In-Process | Milk/ Creamer | Yogurt/ Cultured | Ice Cream | Cheese |
|---|---|---|---|---|---|---|
| Almond | X | X | X | X | X | X |
| Cashew | X | X | X | X | X | X |
| Coconut | X | X | X | X | X | X |
| Soy | X | X | X | X | X | X |
| Oat | X | X | X | X | X | X |
| Mycoprotein | X | X | | | | |

**Note** – "X" annotates successful tests, blank square represents products untested at the time of publication

As a calibration-free technology, the ORACLE avoids error introduced from improper extraction methods and presents a simple SOP, removing human error seen even with fully validated AOAC and ISO techniques. With multiple manual steps that require a relatively high degree of experience to perform correctly, extraction methods naturally have a low reproducibility when comparing labs. The ORACLE is able to improve reproducibility between different users, products and locations, demonstrated in **Table 3**. The data presented is based on a range of dairy products in a 2017 independent evaluation performed by Actalia Cecalait, which concluded that "The ORACLE instrument presents a good performance of repeatability for all the products and below reference method limits" (1).

**Table 3**. Final precision data for ORACLE compared to validated reference method on 30 total dairy samples

| g/100g | Cream | Sour Cream | Yogurt | Cheese | Processed Cheese | Dried Milk | Ice Cream | Milk Dessert | All Samples |
|---|---|---|---|---|---|---|---|---|---|
| n | 4 | 2 | 4 | 6 | 4 | 4 | 2 | 4 | 30 |
| min | 21.87 | 13.90 | 1.04 | 2.28 | 8.55 | 0.42 | 9.18 | 3.02 | 0.42 |
| max | 44.33 | 29.47 | 8.91 | 34.69 | 29.41 | 26.08 | 17.20 | 6.79 | 44.33 |
| Y | 32.90 | 21.54 | 3.58 | 18.55 | 22.14 | 16.70 | 13.18 | 5.48 | 16.80 |
| $S_y$ | 9.66 | 10.87 | 3.61 | 13.43 | 9.54 | 12.14 | 5.81 | 1.78 | 12.53 |
| d | 0.02 | 0.15 | 0.06 | 0.04 | 0.10 | -0.14 | 0.01 | -0.05 | 0.02 |
| $S_d$ | 0.10 | 0.14 | 0.07 | 0.13 | 0.08 | 0.06 | 0.14 | 0.09 | 0.12 |
| $S_{y,x}$ | | | | | | | | | 0.1222 |
| $S_{y,x\%}$ | | | | | | | | | 0.72 |
| Slope | | | | | | | | | 0.999 |
| Bias | | | | | | | | | 0.009 |

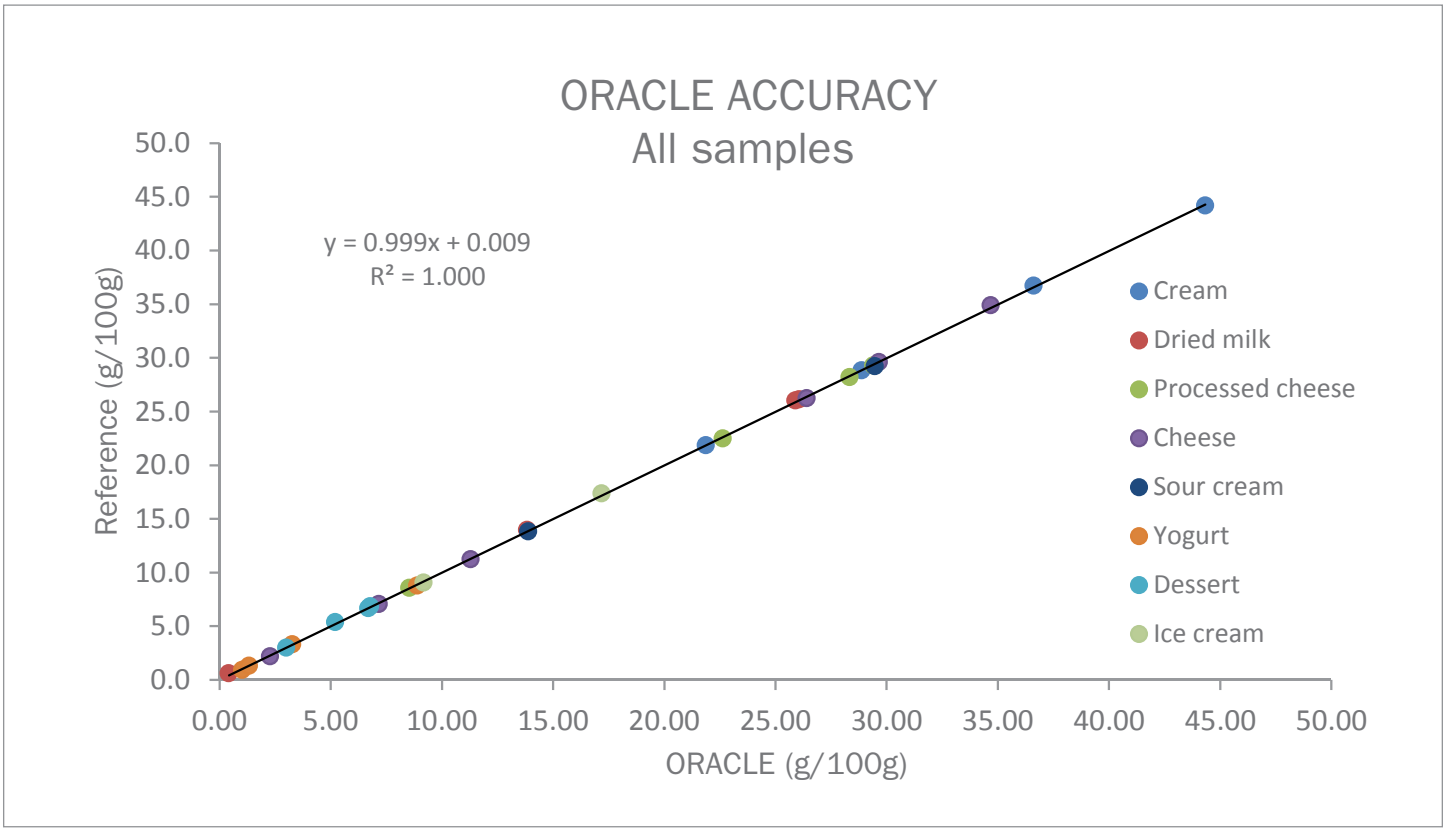


**Figure 2**. Relation between ORACLE and reference results in all in various dairy samples

This ORACLE data stands in stark contrast to the reality of poor reproducibility delivered by the traditional analytical methods. In 2007 the USDA published a study on this issue, comparing results of various routine analytical methods on CRMs across 9 different SIO 17025 accredited laboratories (2).

**Table 4**. Summary of Z-scores[a] for reported nutrient concentrations analyzed in certified reference materials (CRMs)

| Class | Nutrient | Total CRMs | Total Labs | Total Values | Count of 0 to \|1\| | Count of \|1\| to \|2\| | Count of \|2\| to \|3\| | Count of >\|3\| | Count of >\|2\| | Count of >\|3\| |
|---|---|---|---|---|---|---|---|---|---|---|
| Proximates | Moisture | 11 | 7 | 118 | 82 | 22 | 9 | 5 | 11.9 | 4.2 |
| | Protein | 9 | 5 | 106 | 60 | 24 | 12 | 10 | 20.8 | 9.4 |
| | Ash | 11 | 5 | 107 | 55 | 26 | 11 | 15 | 24.3 | 14.0 |
| | Total Fat | 11 | 6 | 129 | 52 | 39 | 15 | 23 | 29.5 | 17.8 |

The fat results, in particular, showed nearly a third of data points falling outside the accepted range of 2 standard deviations, with 18% of all results outside 3 standard deviations. This inability to provide the "correct" answer was seen in many of the analytes, highlighting the trouble with the long-used manual methods of analysis. One corporate study further proved this on the ORACLE for plant-based samples, with ORACLE results maintaining far better consistency than one external lab on repeated batch testing, along with a stronger ability to adapt to product changes. The justification for this company to make the change was based on a less than a 2-year ROI and nearly a million dollars in savings after 10 years in external lab and validation costs alone.

**Protein Testing and a desire for Green Methods**

The ORACLE isn't the only system from CEM which provides these same benefits. Protein is the actual name of the game when it comes to "alternative protein" products and, while this critical analyte may not be as problematic as fat, it still provides many challenges. Kjeldahl digestion and titration has been the focus of protein analysis for over a century, with Dumas techniques showing similar abilities but, unfortunately, similar issues. Both methods are tests for nitrogen that have been adapted to calculate protein based on historical data for amino acid and non-protein nitrogen (NPN) content. However, these values change, and with them the quantity and quality of the protein in food changes as well. This is especially true in plant based and cultivated cell products as they undergo rigorous and constant R&D in the early stages of development and fact findings. Data shows a significantly higher presence and variability of NPN in these products, which will be highlighted in a separate whitepaper by CEM.

Additional tests can be performed to analyze the true protein content of samples, but these tests require twice the time and cost, and doubles the potential for error to be introduced. Building on the proven Udy dye-binding method, CEM created the fully automated rapid protein analyzer, the Sprint. By using a dye-binding molecule that only interacts with protein, not free amino acids or non-protein nitrogen, the Sprint is capable of giving a better protein result for not only the raw ingredients used for plant-based foods, but also the in-process and finished products themselves.

Where the Sprint and ORACLE are best suited for plant-based products applies directly to one of the core values of this new industry: the desire for an environmentally conscious alternative to animal-based food products. Both the ORACLE and Sprint provide the ability to greatly reduce, and in some cases eliminate, the hazardous solvents and waste materials required for traditional fat and protein testing. A single user of CEM products performing approximately 2,000 tests for fat and 2,000 additional tests for protein annually, can remove enough solvent from their tests to fill 2 large oil drums. Continued application of these technologies on a larger, global scale can result in reducing solvent use to a scale measured in Olympic-sized swimming pools on an annual basis.

**Navigating Metals Testing for Plant-Based Products**

Another developing area for plant-based alternative products is the requirement for quality control tests like metals detection. Recent legislation, like Prop 65, seeks to better align heavy metals testing in food and other consumer products. This provides consumers peace of mind and assurance that the food they consume is safe. However, this can be a dual-edged sword for manufacturers of plant-based alternative products. As an example, the amount of mercury present in fish has been a long-standing concern. Plant-based products seek to reduce the mercury concern, with the added benefit of alleviating the environmental impact of commercial fishing, however, it is no secret that plants are known to take up metals from the ground. As a result, plant-based products may have an inherently higher "normal" level of metals than animal-based products. Even further, manufacturers may introduce certain ingredients and additives that could contribute to these elevated levels, all in an effort to change the way a final product looks or tastes, easing the transition for consumers from traditional meat and dairy to plant-based alternatives.

The complexity of navigating FDA and other legislative requirements can be difficult. CEM has been a key collaborator and participant in both AOAC and FDA methodology for sample preparation and analysis of traditional foods. The MARS 6™ microwave digestion system and protocol is referenced in AOAC Method 2015.01 as well as FDA EAM Method 4.7. As an industry leader and innovator, CEM works with many key plant-based companies to consult on the appropriate methods and requirements for metals testing, as well as provide direction on how to avoid critical mistakes that can lead to audits, recalls, and loss of consumer trust.

Below is a brief overview of data gathered by CEM including animal-based ground beef, sausage, chicken, and tuna. These products were chosen because they are readily available and can be purchased in a minimally processed (ground) format, or as a piece, in the case of tuna, which was later ground to obtain a more homogenous sample. The plant-based samples consisted of a ground alternative meat and sausage product, a formed chicken breast alternative and a portion of alternative tuna. The chicken and tuna were ground, similar to their animal-based substitute. A suite of eleven elements were analyzed based upon nutritive, additive, and toxicity in order to provide a broad spectrum of analytes. Two standard reference materials (SRMs) were also prepared and analyzed in order to verify analytical performance. These included NIST reference materials, SRM 1947 Lake Michigan Fish and SRM 1577c bovine liver.

**Table 5:** SRM recovery of eleven elements

| | As % Rec | Cd % Rec | Hg % Rec | Pb % Rec | Ti % Rec | Fe % Rec | Zn % Rec | Se % Rec | Na % Rec | K % Rec | Ca % Rec |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1577C Bovine Liver** | 111.63 | 92.98 | 104.37 | 88.53 | N/A | 91.37 | 91.07 | 108.97 | 96.37 | 104.99 | 105.98 |
| **1947 Lake Michigan Fish Tissue** | 105.02 | N/A | 96 | N/A | N/A | 98.50 | 106.04 | 94.81 | N/A | N/A | N/A |

Data recovery of the SRM elements were all between 88% and 110% recovery, which validates the methodology (both microwave digestion and analysis). In general, the big four toxic elements (Pb, Cd, Hg, and As) are low, as seen in **Table 6**, which is to be expected with consumed goods. Currently the FDA does not establish limits for heavy metals in food; however, if we look at the World Health Organization (WHO) permissible limits for plant materials, we find that lead limits are in the ppm range while cadmium is 1.30 ppm. Neither arsenic nor mercury are listed by the WHO. The plant-based products were found to contain slightly elevated levels as compared to animal-based products due to the uptake of heavy metals from the soil by the plants prior to harvesting. While higher, these levels are well below WHO guidelines (3). Other than cadmium, all big four elements were found to be significantly lower in the plant-based alternative tuna product than in its saltwater fish counterpart, providing a healthier alternative. This is especially true for mercury. It should be noted that some elements, such as iron and zinc, are added to plant-based meats for nutritive content. The salt content (sodium, potassium, and calcium), typically used for seasoning, was similar in both the plant-based and traditional meat samples.

**Table 6**: Metals Analysis of Plant-based and Traditional Meat Samples

| | Big Four Heavy Metals | | | | Nutritive | | | | Added Salts | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | As ppb | Cd ppb | Hg ppb | Pb ppb | Ti ppb | Fe ppb | Zn ppb | Se ppb | Na ppm | K ppm | Ca ppm |
| **Beef** | 2.63 | 0.22 | 1.04 | 4.87 | 31.33 | 24759.21 | 43238.07 | 104.39 | 518.17 | 3399.02 | 46.29 |
| **Plant Beef** | 13.24 | 9.74 | 0.92 | 5.91 | 152.20 | 31509.63 | 41618.37 | 73.62 | 3781.33 | 2692.56 | 223.90 |
| **Chicken** | 0.56 | 0.08 | 0.85 | 2.45 | 47.05 | 5684.12 | 14390.11 | 157.80 | 529.52 | 2848.48 | 64.63 |
| **Plant Chicken Cutlet** | 4.33 | 14.61 | 0.97 | 15.20 | 150.52 | 19617.11 | 5340.75 | 18.59 | 10096.07 | 789.18 | 420.04 |
| **Tuna** | 1624.02 | 10.23 | 245.12 | 2.84 | 50.33 | 3930.20 | 3181.53 | 519.64 | 2060.37 | 2285.59 | 44.39 |
| **Plant Tuna Regular** | 4.64 | 22.6 | 0.33 | 3.58 | 97.37 | 32676.16 | 13783.67 | 72.62 | 3887.42 | 1206.73 | 434.56 |
| **Plant Tuna Herbs & Oil** | 6.04 | 22.60 | 0.75 | 6.31 | 174.14 | 34471.19 | 13851.12 | 65.93 | 4994.33 | 1405.27 | 533.80 |
| **Pork** | 0.54 | 0.12 | 0.62 | 5.94 | 31.75 | 3545.73 | 11710.80 | 141.50 | 425.62 | 3182.14 | 39.14 |
| **Plant Sausage** | 9.06 | 18.12 | 1.83 | 17.60 | 163.71 | 19018.87 | 37634.07 | 54.90 | 7076.8 | 3957.07 | 1469.43 |

## Conclusion

The world of plant-based foods is still one that is adapting and changing, with little established and defined in terms of standards and methods. Even the very definition of “plant-based foods” and “alternative proteins” are not agreed on by the committees and organizations involved with these products. Analytical tests are performed by inference and assumptions, some of which may lead to inaccurate and misleading data. But, as this industry continues to grow, the awareness of these issues also grows, and more data and focus will continue to improve the situations that researchers, manufacturers, and testers of plant-based foods are currently facing. CEM is one of many, whose action and drive is toward a more sustainable future and is committed to supporting the industries that share that common goal.

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